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TERMINAL OXIDATION SYSTEM IN YEAST

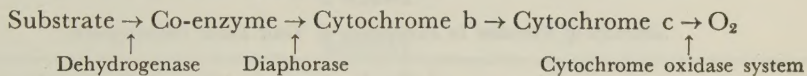
I. ON THE ROLE OF ACTION OF CYTOCHROME b_2

By TATEO YAMANAKA, TAKEKAZU HORIO
AND KAZUO OKUNUKI

(From the Department of Biology, Faculty of Science,
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(Received for publication, September 24, 1957)

In 1937, Yakushiji and Mori (1) reported the extraction and partial purification of cytochrome b from baker's yeast. Using the preparation of cytochrome b, Okunuki and Yakushiji (2) proposed, in 1940, a scheme on the terminal oxidation system as follows:



However, Borei (3) was not able to reproduce the result of Yakushiji and Mori. We (4) have recently succeeded in preparing crystalline cytochrome b_2 from baker's yeast and shown that the findings by them are concerned with nothing except cytochrome b_2 .

In 1954, Appleby and Morton (5) reported the crystallization of lactic dehydrogenase from baker's yeast. The crystalline enzyme involves cytochrome b_2 , while cytochrome b_2 itself does not show any enzymic activity. This fact and the scheme as described above led us to the assumption that cytochrome b_2 may accept an electron from substrates other than lactate.

In fact, we have found that cytochrome b_2 involved in the enzyme purified from baker's yeast accepts an electron from malate and reduced triphosphopyridine nucleotide as well as from lactate. The present paper will deal with such a pivotal action of cytochrome b_2 on the terminal oxidation system in respiring yeast.

MATERIALS AND METHODS

Preparation of Diphosphopyridine Nucleotide (DPN)—DPN of about 90 per cent purity was prepared from baker's yeast (6).

Preparation of Triphosphopyridine Nucleotide (TPN)—TPN of about 80 per cent purity was prepared from DPN and adenosine triphosphate prepared from rabbit muscle (7), using DPN-kinase purified from pigeon liver (8), according to the method of Wang, Kaplan, and Stolzenbach (9).

Preparation of Reduced Triphosphopyridine Nucleotide (TPNH)—TPNH was prepared from TPN using isocitric dehydrogenase purified from pig heart (10), according to the method of Nason and Evans (11).

Preparation of Calcium Phosphate Gel—The gel was made by adding equal volume of

0.66 *M* CaCl₂ to 0.44 *M* (NH₄)₂HPO₄. The mixture was adjusted to pH 8.0 with ammonia water within a short time and centrifuged. The precipitate was washed five times with distilled water and used immediately.

Preparation of Crystalline Cytochrome c from Baker's Yeast—Refer to our previous reports (12, 13).

Preparation of Ferricytochrome c—The solution of cytochrome c was completely oxidized by adding a minimum amount of K₃Fe(CN)₆ and dialyzed overnight against 0.02 *M* phosphate buffer solution of pH 6.0 in a refrigerator.

Preparation of the Enzyme—The enzyme was purified from baker's yeast according to the procedure similar to the method by which Bach, Dixon, and Zervas (14) purified lactic dehydrogenase from the same source. Pressed baker's yeast* (500 g.) was ground with 1 kg. of sea sand or quartz sand for 2 hours in the presence of 5 ml. of 50 per cent sodium lactate. The grinding was carried out in a large mortar with a motor. To the ground matter, 1 liter of distilled water containing 0.5 per cent sodium lactate was added and allowed to stand overnight in a refrigerator with occasional stirring. After removal of most of the precipitated sand by decantation, the extract solution was centrifuged at 14,000 × *g.* for 30 minutes. For the purpose of lowering salt concentration, the supernatant

TABLE I

Summary of Procedure in Purification of Yeast Lactic Dehydrogenase

Fraction	Total volume (ml.)	Q _{MB} (14) (at 30°)	Recovery (%)
Crude extract	700	132	100
Calcium phosphate gel eluate	120	1310	56
Ammonium sulfate fraction (50–55% in saturation)	10	4000	45

was dialyzed overnight against tap water in a refrigerator. To the treated supernatant, calcium phosphate gel (approximately 6.0 g. in dry weight) was added. After 0.5 hour, the gel was collected by centrifugation and washed twice with each 100 ml. of 0.067 *M* phosphate buffer solution (pH 7.2). The washed gel was extracted with 150 ml. of 0.067 *M* phosphate buffer solution (pH 7.2) containing 20 per cent (NH₄)₂SO₄ in saturation. Solid (NH₄)₂SO₄ was added to the extract to 50 per cent in saturation and the precipitate formed was discarded. To the supernatant, saturated (NH₄)₂SO₄ solution was supplemented to 55 per cent in saturation, and then centrifuged at 14,000 × *g.* for 30 minutes. The precipitate was dissolved in 10 ml. of 50 per cent-saturated (NH₄)₂SO₄ solution. The solution showed a fairly high activity of lactic dehydrogenase, Q_{MB} at 30° (14) = 4,000 and was used in this experiment, though the activity could be raised further by fractionation with (NH₄)₂SO₄, since the method for further purification was not established with correct reproducibility owing to its severe lability. The mode of purification is summarized in Table I.

Reagents—Disodium L-malate and trisodium DL-iso-citrate, products of the Nutritional Biochemical Corporation, were used. The malate was purified in the form of its lead salt by the following method: Lead malate precipitated was washed three times with distilled water, and malate was liberated from lead by addition of Na₂SO₄ solution. The liberated malate was used as the purified malate.

* Oriental Yeast Co., Ltd.

Measurement of Dehydrogenase Activities—The activities were measured by following the reduction of ferricytochrome *c* at 550 $m\mu$ in a cuvette (1 cm. in optical length) using Shimadzu photoelectric spectrophotometer, type QB-50. The components were as follows: 2.0 ml. of 0.2 *M* phosphate buffer solution at an experimental pH, 1.0 ml. of ferricytochrome *c* solution, and 0.5 ml. of a substrate. Reaction was started by addition of 0.3 ml. of the enzyme solution which had been diluted to an appropriate concentration. Readings were taken at zero time, before addition of the enzyme solution, and after that, at every 15-second intervals.

Measurement of Absorption Spectrum of the Cytochrome b_2 in the Enzyme Solution—The change of absorption spectrum of the cytochrome b_2 originally contained in the enzyme solution by addition of a substrate was measured at room temperature (about 20°) with the components as follows: 2.0 ml. of oxidized enzyme solution, 1.0 ml. of 0.2 *M* phosphate buffer solution (at an experimental pH), and 0.5 ml. of a substrate.

Preparation of Oxidized Enzyme Solution—The enzyme solution was oxidized by addition of a minimum amount of $K_3Fe(CN)_6$, and the oxidation was confirmed using a hand-spectroscope. To the solution, saturated $(NH_4)_2SO_4$ solution was added to 55 per cent in saturation and centrifuged. The precipitate was washed five times with 55 per cent-saturated $(NH_4)_2SO_4$ solution and dissolved in 50 per cent-saturated $(NH_4)_2SO_4$ solution.

RESULTS

*Reduction of Cytochrome *c* by Various Substrates in the Presence of Oxidized Enzyme Solution*—As presented in Table II, reduction of ferricytochrome *c*

TABLE II

*Reductions of Cytochrome *c* by Various Substrates in the Presence of Oxidized Enzyme*

Components of the reaction mixture were the oxidized enzyme preparation, cytochrome *c* prepared from baker's yeast, 0.1 *M* phosphate buffer solution (pH 7.0), and 0.02 *M* each substrate. Reactions were carried out at room temperature. The results were obtained by observation with a hand-spectroscope.

Substrate added	Strength of reduction of cytochrome <i>c</i>
Lactate	###
TPNH	###
Malate	###
Succinate	—
Fumarate	±
iso-Citrate	±
Citrate	—
Alanine	—
Glutamate	—
Aspartate	—

by various substrates can be observed with a hand-spectroscope in the presence of the diluted, oxidized enzyme. Among the substrates tested, lactate, malate, and TPNH were able to reduce ferricytochrome *c* markedly.

This fact indicates that the enzyme solution also contains other enzymes which transfer electrons from malate and TPNH besides yeast lactic dehydrogenase, to cytochrome c.

In fact, as shown in Fig. 1, ferricytochrome c is spectrophotometrically reduced by addition of each of these substrates, though the reductions by malate and TPNH are approximately one-tenth and three-tenths that by lactate, respectively.

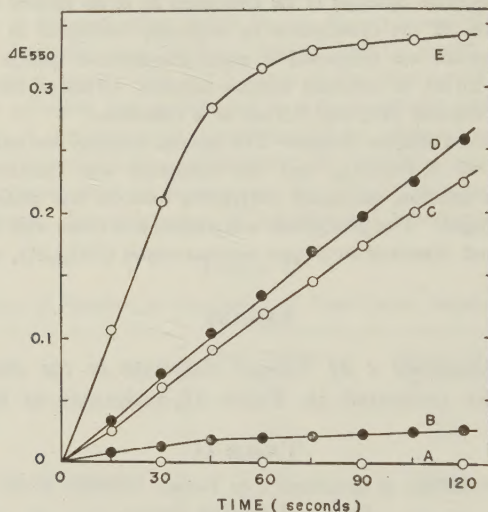


FIG. 1. Reductions of cytochrome c by lactate, malate, and TPNH in the presence of the enzyme preparation.

Reactions were carried out in 0.1 M sodium phosphate buffer solution (pH 8.0) using $0.053 \mu\text{M}$ of cytochrome c (molecular weight calculated as 13,000) crystallized from baker's yeast as an electron acceptor, in the presence of the enzyme preparation which could reduce methylene blue in 165 seconds in both of the cases of malate and TPNH. In the case of lactate, the enzyme preparation was used in 1/10 concentration as in the former cases. (A), $+0.19 \mu\text{M}$ of TPNH in the absence of the enzyme preparation; (B), no substrate; (C), $+50 \mu\text{M}$ of L-malate; (D), $+50 \mu\text{M}$ of DL-lactate; (E), $+0.19 \mu\text{M}$ of TPNH.

These facts might suggest the possibility that the enzyme preparation contained TPNH-cytochrome c reductase and "malic" enzyme (TPN-linked), since the reductase has been reported to transfer an electron from TPNH to ferricytochrome c.

Reductions of the Cytochrome b_2 in the Enzyme Preparation by Lactate, Malate, and TPNH—As shown in Figs. 2 and 3, the α -band of the cytochrome b_2 present in the enzyme preparation appears at $557 \text{ m}\mu$ immediately after additions of lactate, malate, and TPNH. This fact indicates that in the enzyme preparation there are enzymes which transfer an electron from lactate, malate, and

TPNH to ferricytochrome b_2 . The enzyme is extremely labile against dialysis and, therefore, it was washed by repeating precipitation and solubilization with alternative use of 55 and 50 per cent ammonium sulfate in saturation for the purpose of removal of a certain cofactor such as TPN which might be contaminating the enzyme solution. But the washed enzyme preparation presented the same results.

As shown in Fig. 2, the increment in optical density at 557 $m\mu$ (α -band of cytochrome b_2) in the presence of malate was lower than that in the

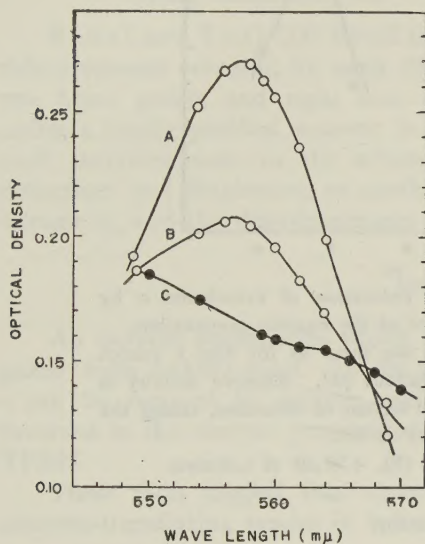


FIG. 2. Reduction of the cytochrome b_2 contained in the enzyme preparation by lactate, and malate.

Absorption spectra were measured in one-cm. optical length at 20° immediately after addition of each substrate to the common composition containing the enzyme preparation and 0.057 M sodium phosphate buffer solution (pH 6.0).

(A), +50 μM of DL-lactate; (B), +50 μM of L-malate; (C), control (to confirm the cytochrome b_2 contained in it to be at oxidized form)

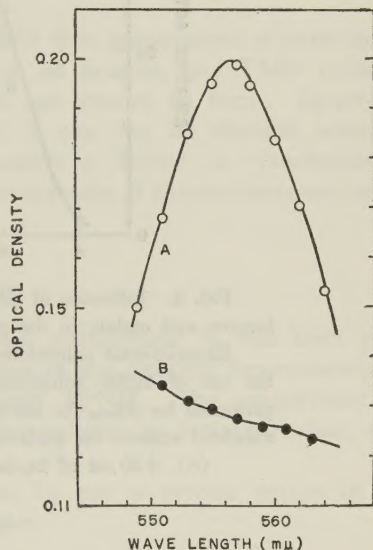


FIG. 3. Reduction of the cytochrome b_2 contained in the enzyme preparation by TPNH.

The experimental conditions were the same as for Fig. 2, except the use of buffer solution (pH 8.0).

(A), +0.18 μM of TPNH; (B), control.

presence of lactate of the same concentration. Therefore, it was thought that a small amount of lactate might be present in the reagent used as malate. However, the same results were obtained with the malate purified through its lead salt. Fig. 3 shows the reduction of the cytochrome b_2 by the addition of TPNH.

In Fig. 4, the influences of pH are shown on the reduction of ferricytochrome c by lactate and malate. The former reaction has the optimal pH at 6.5 and the latter at 8.0. This difference shows that the reduction of

cytochrome c by the reagent used as malate is not the result from the lactate contaminating the reagent.

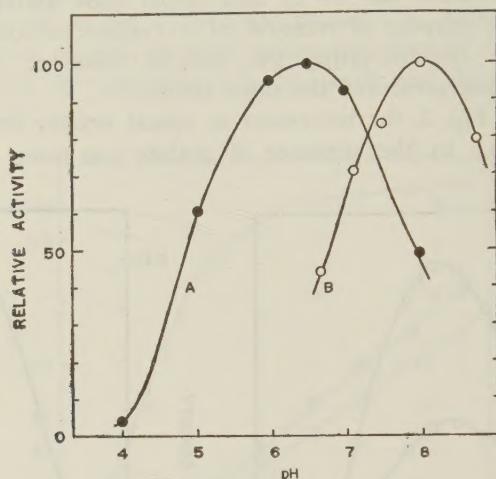


FIG. 4. Influence of pH on reductions of cytochrome c by lactate, and malate in the presence of the enzyme preparation.

Experimental conditions were the same as for Fig. 1 except the use of buffer solutions of various pH. Relative activity is presented by ΔE_{550} for the first 30 second of reduction, taking the maximal activity for each substrate as 100.

(A), +50 μ M of DL-lactate; (B), +50 μ M of L-malate

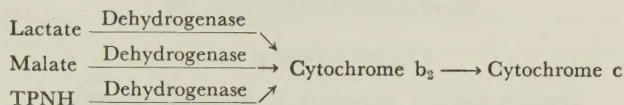
DISCUSSION

It is well known that there is an enzyme in baker's yeast which dehydrogenates lactate and transfers the electron to ferricytochrome c, and the enzyme of such an action was called yeast lactic dehydrogenase. Appleby and Morton (5) reported that the enzyme is crystallizable and the crystalline enzyme involves FMN and cytochrome b_2 . Recently, we (4) have succeeded in the preparation of crystalline cytochrome b_2 from baker's yeast which does not have such enzymic activity and FMN. This fact suggests that the yeast lactic dehydrogenase may be a complex of cytochrome b_2 and a flavine enzyme. On the other hand, it is not known how lactate is produced in baker's yeast, since the DPN-linked lactic dehydrogenase such as that generally found from animal sources has not yet been detected. These facts lead us to a doubt whether cytochrome b_2 may not exist as an electron carrier from only lactate to cytochrome c.

Actually, the results shown in Figs. 1, 2, and 3 indicate that cytochrome b_2 is an electron carrier from malate and TPNH as well as from lactate, to cytochrome c. It also seems likely from the result of Fig. 4 that the reactions from lactate and that from malate to cytochrome c are catalyzed by dehydrogenases different from each other in respect to their optimal pH.

It is still uncertain whether or not the dehydrogenases of malate and TPNH are conjoint to cytochrome b_2 as well as the yeast lactic dehydrogenase crystallized by Appleby and Morton, since we have not yet succeeded in further purification of the enzyme.

To sum up, an electron-transferring system could be presented as in the following scheme:



Boeri and Tosi (15) found that their best preparation of yeast lactic dehydrogenase contains, for each 230,000 g. of protein, one FMN residue, one heme group, and eight iron atoms not bound to heme. However, unless a highly purified enzyme is used, it can not be decided whether each dehydrogenase in the scheme involves a flavine as cytochrome c reductases and diaphorase, or another flavoprotein is situated between cytochrome b_2 and the dehydrogenases.

SUMMARY

An enzyme preparation which contains cytochrome b_2 has been prepared from baker's yeast. In the presence of the enzyme, ferricytochrome c can be reduced by lactate, malate, and TPNH. The cytochrome b_2 involved in the enzyme preparation is also reduced by lactate, malate, and TPNH.

These facts suggest that cytochrome b_2 has a pivotal action in an electron-transferring system in baker's yeast.

We would like to express our hearty thanks to Oriental Yeast Co., Ltd. for their supply of pressed baker's yeast, and to Mr. K. Fujii* for his guidance. We should like to thank our colleagues Messrs. T. Higashi, M. Nozaki, J. Yamashita and H. Mizushima for helpful discussions during the course of this work.

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MICROBIOLOGICAL DEGRADATION OF BILE ACIDS

XVII. SUPPLEMENTARY STUDIES ON CHOLIC ACID DEGRADATION BY CORYNEBACTERIUM SP.

By KEIZO TAMAKI

(From the Department of Biochemistry, Okayama University Medical School, Okayama)

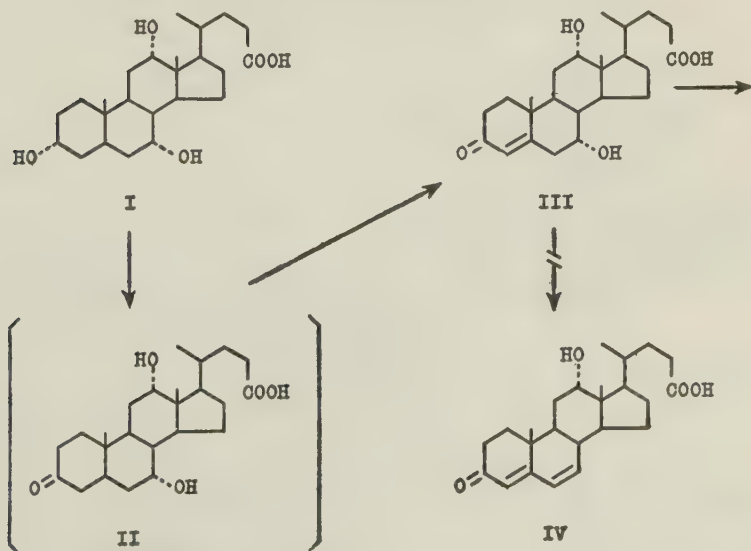
(Received for publication, December 3, 1957)

In the previous papers, it has been demonstrated that a soil bacterium, cultured in a synthetic medium containing cholic acid (Fig. 1, I) as the sole source of carbon, converts this acid to a new unsaturated acid (I), 7 α , 12 α -dihydroxy-3-oxo- Δ^4 -cholenic acid (III), and subsequently this microorganism is identified as *Corynebacterium* sp. (2).

In a proposed pathway (1) for the conversion of cholic acid (I) by *Corynebacterium* sp. to the acid III, as seen in Diagram 1, an existence of

DIAGRAM 1

Proposed degradation pathway of cholic acid by *Corynebacterium* sp.



7 α , 12 α -dihydroxy-3-oxocholanic acid (II) was expected as the primary oxidation intermediate of cholic acid. In a microbiological degradation of cholic acid by *Streptomyces gelaticus* 1164 to 7 α -hydroxy-3,12-dioxo- Δ^4 -bisor-

cholenic acid, various intermediates were isolated by using a sythetic medium containing glucose and cholic acid as the carbon sources (3).

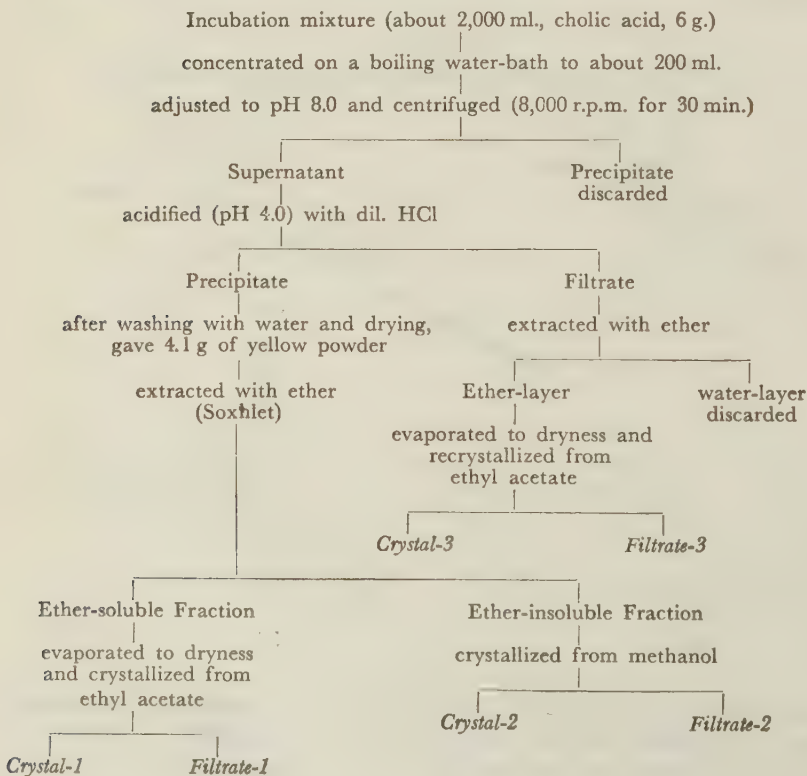
The present experiment was carried out, in an attempt to obtain the expected acid II by using the above glucose-cholate medium but the isolation the asid II as crystals was not in a success. While 7 α , 12 α -dihydroxy-3-oxo- Δ^4 -cholenic acid (III) and 12 α -hydroxy-3-oxo- $\Delta^{4,6}$ -choladienic acid (IV) were isolated as the degradation products of cholic acid and unchanged cholic acid was recovered. As discussed later, however, the acid IV isolated here may not be a microbiological degradation product of cholic acid, but it is probably an artificial transformation product of the acid III.

EXPERIMENTAL

*Separation of 7 α , 12 α -Dihydroxy-3-oxo- Δ^4 -cholenic Acid (III), 12 α -Hydroxy-3-oxo- $\Delta^{4,6}$ -choladienic Acid (IV) and Cholic Acid (I) from the Glucose-Cholate Culture with *Corynebacterium Sp.*—A synthetic medium (3) which was used for the cholic acid degradation by *S. gelaticus* 1164 was adopted in this experiment.*

SCHEME 1

Separation of Cholic Acid Metabolites by Corynebacterium Sp.



6 g. of cholic acid was used and the inoculated medium with *Corynebacterium* sp. was incubated at 37° for 6 days. At the end of the incubation period the incubation mixture was treated as shown in Scheme 1, and the various acids or esters were isolated and identified from the following fractions.

Crystal-1: Further recrystallization from ethyl alcohol gave 0.2 g. of pale yellow prismatic needles, m.p. 249–252° and $\lambda_{\text{max}}^{\text{alc}}$ 284 m μ (log ϵ , 4.42). Identity with an authentic 12 α -hydroxy-3-oxo- $\Delta^{4,6}$ -choladienic acid (4) was demonstrated by mixed melting point determination and infrared comparison.

Filtrate-1: After evaporation of the solvent, the resulting yellow residue (1.2 g.) was esterified with diazomethane in ether and chromatographed by the same manner described for the next Filtrate-2. Various eluents with

TABLE I
Chromatographic Details for the Filtrate-2 in Scheme 1

Fraction number	Solvents of elution (ml.)	Yield (mg.)	Identified substances	m.p.
1,2,3,4	Ether (14 ml.)	None	—	—
5,6	„ (,,)	Trace	—	—
7,8,6	„ (,,)	150	Methyl 12 α -hydroxy-3-oxo- $\Delta^{4,6}$ -choladienate*	182–184°
10,11,15	„ (,,)	Trace	—	—
13,14,15	„ (,,)	None	—	—
16	Ether: methanol 9 : 1 (,,)	Trace	—	—
17	„ (,,)	430	(Oily gum)	—
18	„ (,,)	70	Methyl 7 α ,12 α -dihydroxy-3-oxo- Δ^4 -cholenate**	177–179°
19,20	„ (,,)	20	Methyl cholate***	152–154°

* Recrystallized from ethyl acetate-petroleum ether and then methanol.

** Recrystallized from ethyl acetate-ether. (Analysis Calcd. for $\text{C}_{25}\text{H}_{38}\text{O}_5$: C, 71.74; H, 9.15. Found: C, 71.47; H, 9.25.)

*** Recrystallized from ethyl acetate-petroleum ether.

ether were obtained, but only 0.1 g. of methyl 12 α -hydroxy-3-oxo- $\Delta^{4,6}$ -choladienate with m.p. 183–185° was obtained as crystals from the earlier eluents.

Analysis Calcd. for $\text{C}_{25}\text{H}_{36}\text{O}_4$: C, 74.96; H, 9.06
Found C, 74.84; H, 8.85

Crystal-2: Further recrystallization from ethyl alcohol gave 2.0 g. of tetrahedral crystals with m.p. 196–198°. Identity with an authentic cholic acid with m.p. 198–199° was demonstrated by mixed melting point determination.

Filtrate-2: After evaporation of the solvent, the resulting residue (0.9 g.)

was esterified with diazomethane in ether. After drying, 20 ml. of ether was added, and soon a crystal with m.p. 146–154° and $\lambda_{\text{max}}^{\text{alc.}}$ 242 m μ was separated out. The crystal was filtered and the ether filtrate was used for the next chromatographic analysis. After recrystallization from ethyl acetate and then ethyl acetate-ether, the melting point raised to 177–179°. Identity with methyl 7 α , 1 α -dihydroxy-3-oxo- Δ^4 -cholenate (1) was demonstrated by mixed melting point determination and infrared comparison.

The ether filtrate was poured onto the 25 g. of alumina column. Several fractions were eluted with various solvents as shown in Table I, and three esters were isolated and identified by mixed melting point determination and infrared comparison.

Crystal-3: Further recrystallization from ethyl alcohol gave 0.1 g. of cholic acid with m.p. 195–199°.

Filtrate-3: After evaporation of the solvent, the resulting residue (1.1 g.) was esterified with diazomethane and chromatographed by the same manner as described for the above *Filtrate-2*. 120 mg. of methyl 12 α -hydroxy-3-oxo- $\Delta^{4,6}$ -choladienate and 340 mg. of oily gum were obtained.

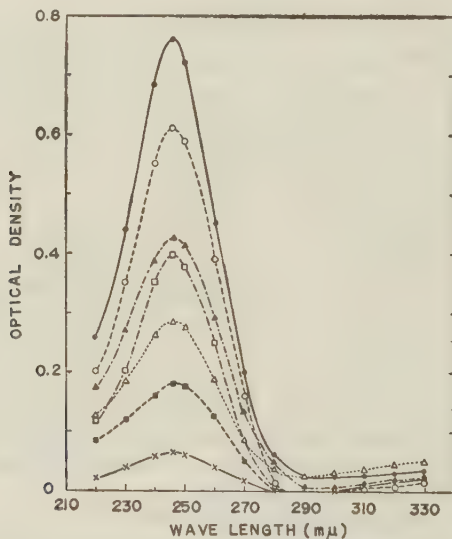


FIG. 1. Ultraviolet absorption spectra of the diluted culture supernatants obtained after cultivation of *Corynebacterium* sp. in a glucose-cholate medium at 37° for the following incubation periods: —x—, 1 day; ...Δ..., 2 days, - -□- - , 3 days, --○--, 4 days; —●—, 5 days, --▲-- , 7 days, --■-- , 9 days. The 1–4 days' incubation mixture were diluted in 1:50 with water, while 5–9 days' incubation mixture in 1:100.

Both oily gums from the *Filtrate-2* and *3* gave no characteristic ultraviolet absorption in the region of 220 to 330 m μ and indicated a positive Zimmermann's test (5) which is known to be specific for bile acids containing the 3-oxo group. So the both gums were combined, chromato-

graphed on alumina and designed to isolate methyl 7α , 12α -dihydroxy-3-oxocholanoate from those gums, but this attempt was unsuccessful.

Spectrophotometric Analyses of Degradation Products of Cholic Acid in Corynebacterium sp. Culture.—A synthetic glucose-cholate medium which was described in a previous paper (3) was used in this experiment. After inoculation of *Corynebacterium* sp., an aliquot of the culture medium was diluted (1:50 or 1:100) with water and centrifuged at 4,000 r.p.m. for 15 minutes. The ultraviolet absorption spectra of the supernatants were measured by Beckman spectrophotometer, type DK I. The data were given in Fig. 1.

As seen in Fig. 1, there is only one peak at $246\text{ m}\mu$ which is the specific absorption maximum for the acid III. But no absorption is observed at $290\text{ m}\mu$ which is specific for the acid IV. This result suggests that the acid IV obtained by the present experiments may not be produced by the microorganism used, but is an artificial product accompanied by the isolation process. In support of this suggestion it may be recalled that a 7α -hydroxy- Δ^4 -3-ketone structure is easily converted to a $\Delta^{4,6}$ -3-ketone by mineral acid or caustic alkali treatment (6).

SUMMARY

1. Microbiological transformation of cholic acid to 7α , 12α -dihydroxy-3-oxo- Δ^4 -cholenic acid by *Corynebacterium* sp. may take place either in a synthetic medium containing cholic acid as the sole source of carbon or in a medium containing glucose in addition to cholic acid as the carbon sources.

2. 7α , 12α -Dihydroxy-3-oxo- Δ^4 -cholenic acid (III) 12α -hydroxy-3-oxo- $\Delta^{4,6}$ -choladienic acid (IV) were obtained as the degradation products of cholic acid by *Corynebacterium* sp., but the ultraviolet absorption data of the culture suggest that a conversion of the acid III to IV may be not an enzymatic reaction.

The author wishes to express his sincere thanks to Prof. T. Shimizu, Prof. S. Mizushima and Dr. S. Hayakawa for their kind guidance throughout this research, and to Mr. Y. Matsui of Research Laboratory, Shionogi & Co., Ltd. for his help in measuring the infrared absorption spectra.

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CRYSTALLINE BACTERIAL PROTEINASE

III. COMPARISON OF CRYSTALLINE PROTEINASE OF *BACILLUS SUBTILIS* WITH CRYSTALLINE TRYPSIN

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Several properties of crystalline proteinase of *Bac. subtilis* N' (1), its stability and proteolytic and esterase activities at various pH, were reported in the preceding paper (2). Since the enzyme was found to be one of neutral proteinase, a few properties of the enzyme, especially its stability and proteolytic activity measured by various assay methods, were compared with those of most well-known neutral proteinase, trypsin. The results obtained will be presented here.

MATERIALS AND METHODS

Crystalline Bacterial Proteinase—Crystalline proteinase of *Bac. subtilis* N', BPN', described in the preceding report (2) was used.

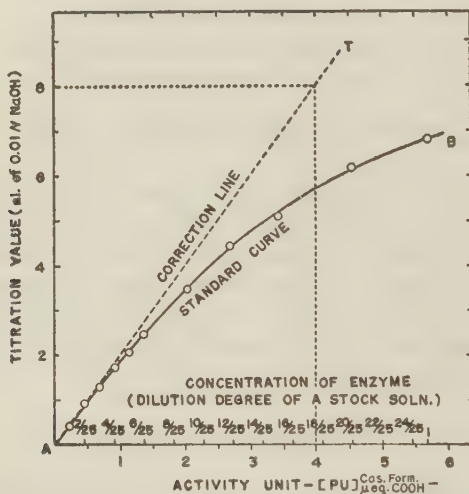
Crystalline Trypsin—A commercial preparation "Trypsin Mochida" of Mochida Pharmaceutical Co. (Tokyo, Japan) was used. Though the preparation contained a small amount of non-protein substance, presumably autodigestion product of the enzyme protein, it was confirmed that specific activity of the preparation based on protein nitrogen was almost the same as the preparation obtained by the method of Kunitz and Northrop (3).

Assay for Protein Digestion Activity—Methods for assaying the activity producing non-protein digestion product from casein using 275 m μ extinction or Folin color reaction were described in the previous reports (1, 2). As the substrate, besides casein in the above method, crystalline bovine hemoglobin (4) was also used after denaturation with 6 M urea. In this case, concentration of hemoglobin and urea was adjusted in such a way as to be 1 per cent and 3 M respectively in the reaction mixture, and the reagent A (0.44 M trichloroacetic acid) was only used as protein precipitation agent. The unit of proteolytic activity was based on the microgram of tyrosine-equivalent per minute's reaction at 30°, pH 7.5, in the same way as in the casein-275 m μ method (1), and represented by $[\text{PU}]_{\mu\text{g. tyr.}}^{\text{cas. 275, A}}$ or $[\text{PU}]_{\mu\text{g. tyr.}}^{\text{cas. 275, B}}$ in the casein-275 m μ method using the precipitation reagent A or B respectively, by $[\text{PU}]_{\mu\text{g. tyr.}}^{\text{cas. Fol. A}}$ or $[\text{PU}]_{\mu\text{g. tyr.}}^{\text{cas. Fol. B}}$ in the casein-Folin

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method with reagent A or B, and by $[\text{PU}]_{\mu\text{g. tyr.}}^{\text{Hb. Fol.}}$ in the hemoglobin-Folin method using the reagent A.

Assay for Peptide-Bond Hydrolysing Activity—Using casein and gelatin as a substrate and 0.02 M sodium phosphate of pH 7.5 as buffer solution, activity of peptide bond hydrolysis was measured by formol titration in the same way as described in the preceding paper (2). Since the titration volume (ml. of 0.01 N NaOH) were not in parallel with the enzyme concentration, each measured value was corrected by the standard curve. As an example, the standard curve obtained in the case of casein substrate is shown in Fig. 1.



the above condition, the unit of the original enzyme solution could be calculated by the following equation:

$$[\text{FP}]_{\text{mg. cas.}} = 6 (\text{mg. of casein in the above solution}) \times \frac{D (\text{dilution number})}{10 (\text{minutes of reaction})}$$

Gelatin-liquefying activity was determined as follows: One ml. of the enzyme solution was reacted with 5 ml. of 6 per cent gelatin solution containing phosphate buffer of pH 7.5 for 10 minutes at 30°, and just sufficient concentration of enzyme to bring the solution not coagulated after 10 minutes' cooling in ice water was found. The unit, [LP]mg. gel., was defined as the activity which brought 1 mg. of gelatin to the state not coagulated at 0° in 1 minute's reaction at 30°. $[\text{LP}]_{\text{mg. gel.}} = 50 \times 6 \times \frac{D}{10}$

Milk-clotting activity was measured at 30° for 10 minutes using 1 ml. of enzyme solution and 10 ml. of 13 per cent milk powder solution containing 0.02 *M* sodium acetate buffer at pH 5.8 in the presence of 0.02 *M* calcium acetate or pH 5.3 in the absence of calcium salt. The end point of the reaction was taken as the appearance of casein particle in the film formed on the surface of a glass tube which was sloped at the angle of 30° and rotated gently. The activity unit, $[\text{CP}]_{\text{ml. milk}}^{\text{pH 5.8, Ca}}$ or $[\text{CP}]_{\text{ml. milk}}^{\text{pH 5.3}}$, was defined as the minimum activity which made 1 ml. of 13 per cent solution of milk powder (concentration of component correspond to usual milk) clotted at 30° in 1 minute's reaction in the presence of calcium ion at pH 5.8 or in the absence of calcium at pH 5.3. $[\text{CP}]_{\text{ml. milk}} = 10 \times \frac{D}{10}$

Course of Hydrolysis of Proteins—Twenty ml. of 2.5 per cent casein or gelatin solution (corrected for moisture content) containing 1/30 *M* sodium phosphate buffer of pH 7.8, was mixed with 5 ml. of 0.075–0.65 mg. PN/ml. of BPN' or 150 mg. PN/ml. of crystalline trypsin, and allowed to react at 30°. At suitable intervals during the reaction period, 3 ml. of the reaction mixture was transferred into 3 ml. of 40 per cent formol solution and electrometrically titrated with 0.01 *N* sodium hydroxide as mentioned before. Percentage hydrolysis of peptide bonds in casein or gelatin molecule based on the similar titration values after acid hydrolysis of the same concentration of the proteins, *i.e.* the proteins were hydrolyzed with 6 *N* hydrochloric acid at 105° for 24 hours, the product was dried in vacuum, dissolved in minimum volume of water, neutralized to pH 8.3 with 0.5 *N* sodium hydroxide, added with the same volume of 40 per cent formol solution of pH 8.3, and titrated with 0.01 *N* sodium hydroxide to the same pH.

RESULTS AND DISCUSSION

Stability of BPN' and Trypsin—In the preceding report (2) it was shown that BPN' was fairly stable in neutral solution containing salts below 40°. On the other hand, it is a well known fact that trypsin is rapidly inactivated in neutral solution owing to its autodigestion. Though it has been reported that trypsin is protected from inactivation at neutral pH by the presence of calcium salt, the stability of the above two proteinases in the present investigation was compared in neutral sodium phosphate buffer solution which is usually used for the reaction of both enzymes. The results are shown in Fig. 2. As shown in this figure, the time required to

decrease one-half of the original enzyme activity at 38° was about 35 hours in the case of 0.1 per cent BPN' and only 1.2 hours in the same concentration of trypsin. In the case of 0.01 per cent solution of BPN', only 5 per cent of the activity decreased after 35 hours, while trypsin in the same concentration was inactivated to 50 per cent after 3.5 hours.

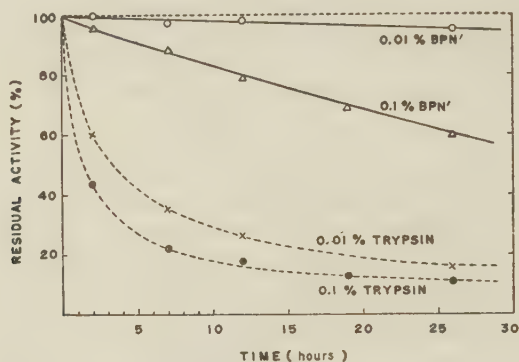


FIG. 2. Course of inactivation of BPN' and trypsin.
Conditions—38°, in 0.05 M sodium phosphate buffer, pH 7.5.

Activity by Different Methods—Many different methods based on different principle have been presented for the assay of proteolytic enzyme and the nature of a proteolytic enzyme has been determined by the comparison of activity measured by the above different methods. In order to make clear the difference in enzymatic nature between BPN' and trypsin, the activities of the two enzymes were compared using several representative methods. The results are summarized in Table I, in which all the unit of proteolytic activity was expressed per mg. of protein nitrogen of the enzymes. As shown in the last column of the table, by casein digesting activity such as casein-275 m μ and casein-Folin method, BPN' showed 2–2.5 times as strong an activity as trypsin. In the above two methods, the activity ratio of BPN' and trypsin was always larger in the methods using the precipitation reagent A than in those using the reagent B. This result is due to the following facts: (1) Casein is hardly digested by trypsin to the state not precipitated with concentrated trichloroacetic acid contained in the reagent A but rather easily to the state not precipitated with diluted one contained in the reagent B. (2) On the contrary, many denatured proteins are easily digested by proteinase of *Bac. subtilis* to the state not precipitated even with concentrated trichloroacetic acid as already reported by the authors (7, 8).

From the result obtained by the method using hemoglobin as substrate, it was shown that BPN' digested this protein twice as easier than casein, but trypsin did only about 1.6 times easier. From this fact it was shown that hemoglobin was a very susceptible substrate to BPN' but not so much

to trypsin. Since the Frud-Gross method shows the activity of the enzyme by which casein was digested to the completely soluble state with the protein precipitation reagent, much difference of this activity was observed between BPN' and trypsin.

By the formol titration method, BPN' was shown to have about twice as much activity of peptide bond hydrolysis than trypsin, as well as protein digestion activity. However, when gelatin was used as a substrate, it was somewhat smaller than in the case of casein substrate. Milk clotting activity of BPN' was much stronger than that of trypsin.

TABLE I
Comparison of Activity Measured by Various Methods between Crystalline Proteinase of Bac. Subtilis N' (BPN') and Trypsin

Principle	Method	Activity unit	BPN'	Trypsin	Ratio
Digestion of protein	Casein-275 m μ , by reagent B	[PU] ^{cas. 275, B} / _{μg. tyr.} / μ g PN	12.4	6.0	2.1
	Casein-275 m μ , by reagent A	[PU] ^{cas. 275, A} / _{μg. tyr.} / μ g PN	10.5	4.2	2.5
	Casein-Folin, by reagent B	[PU] ^{cas. Fol., B} / _{μg. tyr.} / μ g PN	8.2	4.2	1.95
	Casein-Folin, by reagent A	[PU] ^{cas. Fol., A} / _{μg. tyr.} / μ g PN	6.4	2.7	2.4
	Hemolobin-Folin, by reagent A	[PU] ^{Hb. Fol., A} / _{μg. tyr.} / μ g PN	12.1	4.4	2.8
	Frud-Gross	[FP] _{mg. cas./mg. PN}	3.4	1.1	3.1
	Gelatin-liquefying	[FP] _{mg. gel./mg. PN}	4.6	2.4	1.9
Peptide hydrolysis	Casein-formol elect. titration	[PU] ^{cas. form.E} / _{μ eq. COOH} /mg. PN	65	28	2.3
	Gelatin-formol elect. titration	[PU] ^{gel. form.E} / _{μ eq. COOH} /mg. PN	120	62	1.9
Milk clotting	In the presence of Ca ⁺⁺ , pH 5.8	[CP] ^{Ca⁺⁺, 5.8} / _{ml. milk} /mg. PN	180	22	8.2
	In the absenae of Ca ⁺⁺ , pH 5.3	[CP] ^{5.3} / _{ml. milk} /mg. PN	76	7.8	9.7

Hydrolysing Course of Proteins—The course of hydrolysis of casein and gelatin with trypsin and different concentration of BPN' at 30° are shown in Fig. 3. Concentration of trypsin, 180 μ g. PN/ml. in the reaction mixture, was that showing almost the same initial activity as the most concentrated BPN', 35 μ g. PN/ml. Though the pH of the reaction mixture in this experiment dropped during the reaction (from 7.8 to 6.8 at most) on account of the production of peptides and amino acids, it was kept within fairly active pH for both proteinases. As shown in the figure two proteins, casein and gelatin, were rapidly hydrolyzed at first then gradually, and definite extent

of hydrolysis was difficult to be observed by the action of BPN'. However, with very concentrated BPN', *e.g.* 35 μg . PN of enzyme per ml. in the reaction mixture, they were only slightly hydrolyzed after reaction for 21 hours, and therefore the value of hydrolysis at this point was regarded as the extent of hydrolysis. This extent of hydrolysis was 35 per cent for casein and 28 per cent for gelatin. From the digestion products of the above

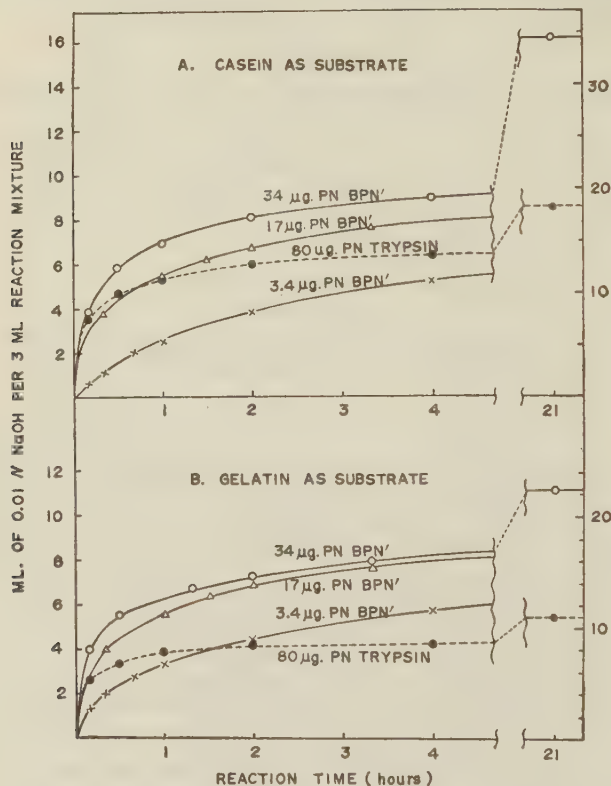


FIG. 3. Course of hydrolysis of casein and gelatin with various concentration of BPN' and trypsin.

Concentration of enzyme in the figure—per ml. reaction mixture. Reaction—2.5 per cent substrate in the reaction mix., at 30° pH 7.8 (initial) to 6.6–7.0 (final). Per cent of hydrolysis—based upon the titration value after hydrolysis with 6 *N* hydrochloric acid.

reaction mixture, many free amino acids were detected by paper chromatography. In the case of trypsin, the above proteins were rapidly hydrolyzed to the same extent as well as BPN', but further hydrolysis was slight, and extent of hydrolysis was 18 per cent for casein and 11 per cent for gelatin. The above larger extent of hydrolysis of the proteins with BPN' is thought to be due to the wide specificity of this enzyme. It was shown that amino

and carboxyl side of almost all of acidic and neutral amino acid residues in a protein could be hydrolyzed by this proteinase (9), while it is a well-known fact that only carboxyl side of a few basic amino acid residues can be easily hydrolyzed by trypsin.

SUMMARY

Stability at neutral pH, proteolytic activity measured by various assay methods, and course of digestion of casein and gelatin were compared between crystalline proteinase of *Bac. subtilis*, BPN', and crystalline trypsin. The time required to decrease 0.1 per cent solution of BPN' was about 30 times longer than that of trypsin. Activity of BPN' measured by casein or hemoglobin digestion methods, gelatin liquefying method and formol titration method using casein and gelatin as substrate, was 1.9-3.3 times stronger than that of trypsin, and milk clotting activity of BPN' was about 10 times stronger than trypsin.

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A NEW COLORIMETRIC DETERMINATION METHOD OF SPHINGOSINE BASE IN LIPIDS

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Though it was a long time since the discovery of sphingolipids, the determination of sphingosine in lipids has been only reported by McKibbin and Taylor (1) (1949) up to the present. The procedure consists of three steps: hydrolysis with $\text{Ba}(\text{OH})_2$ and HCl , extraction of hydrolysate with chloroform and determination of chloroform extractable nitrogen. This method, however, could not be specific for sphingosine base, because many kinds of other amines might be extracted together with chloroform as contaminative substances. Therefore, it would be desirable to find a more suitable method which is used only for the determination of sphingosine base.

Ohno (2) (1944) has already reported that a higher fatty aldehyde is produced from sphingosine by oxidation with lead tetraacetate.

In the present paper a new colorimetric method is reported. The method applies Ohno's method and plasmal reaction (3) (1938-39) and determines fatty aldehyde derived from sphingosine base by oxidation with lead tetraacetate.

METHODS

Reagents: Ethanol (95 per cent)

Chloroform (dehydrated and distilled)

Saturated barium hydroxide solution

Conc. hydrochloric acid

Benzene (dehydrated and distilled)

Lead tetraacetate (this reagent must be kept dry) (L. T. A.)

Glycerol (dehydrated and distilled)

Petroleum ether (b. p. 30° - 60°)

Glacial acetic acid

Schiff's reagent: 1 g. of fuchsin is dissolved in 700 ml. of boiling water and after cooling to room temperature, the solution is filtered into 1 liter of brown coloured measuring flask. Then, 50 ml. of 2 N HCl and 5 g. of NaHSO_3 are added into the flask, mixed thoroughly and finally the solution is made 1 liter by addition of water. This reagent is allowed to stand in dark and cold place till the solution is decolorized completely. This colourless solution is used in the experiment.

SO_2 -water: This reagent is prepared as Schiff's reagent is done except that

fuchsin is not contained.

iso-Amylalcohol (distilled)

All reagents used ought not to contain aldehyde.

Analytical Procedure—Freshly prepared and weighed tissues were put into a mortar and ground together with dry ice. Ethanol with the ratio of roughly 50 ml. per g. of fresh weighed tissue was added to the frozen powder. It was, then, refluxed for 1 hour and filtered. The residues were again extracted with ethanol in the same way. The combined extracts were evaporated to dryness under reduced pressure in the stream of nitrogen. The residues were dissolved in an appropriate volume of chloroform. Aliquots of the solution containing 0.3–2.0 mg. of sphingolipids were taken into a round flask and the solvent was evaporated to dryness on hot water bath under reduced pressure. Then, the sphingolipids were hydrolysed with 10 ml. of $\text{Ba}(\text{OH})_2$ by heating in the boiling water bath under reflux 6 hours. 1 ml. of conc. HCl was then added and the hydrolysis was continued for 1 hour. The hydrolysate was cooled to room temperature, made alkaline with NaOH, sphingosine base was extracted three times with chloroform separately and its totaling volume was 30 ml. The combined chloroform extracts were evaporated to complete dryness on a warm water bath under reduced pressure. The residues were redissolved in benzene, filtered if necessary, and the benzene solution was quantitatively transferred into a volumetric flask.

Aliquots of this solution containing 0.03–0.5 mg. of sphingosine base were taken into test tube. Then, L. T. A. crystals (about ten mg.) were added and the oxidation was carried out for 10 minutes at 75°. A few drops of glycerol were added and the heating was continued until the complete reduction of excess of L. T. A. After being cooled to room temperature, 20 ml. of petroleum ether and 1 ml. of water were added and mixed. The ether layer was transferred into another tube. The extraction was repeated again with 5 ml. of petroleum ether. The combined extracts were evaporated to dryness under reduced pressure. The residues were dissolved in 1 ml. of glacial acetic acid, and then 10 ml. of Schiff's reagent was added, closed with glass stopper, and stood for 24 hours. 10 ml. of SO_2 -water was added to it and well mixed. Then, 10 ml. of *iso*-amylalcohol was added and vigorously shaken. A few minutes later the solution was transferred into a centrifuge tube and centrifuged for 10 minutes. The colour of *iso*-amylalcohol layer was colorimetrically determined with spectrophotometer or stepphotometer. The extinction obtained showed E_1 . The blank determination was carried out in the same way without L. T. A. The extinction of it showed E_2 . Then, E , the extinction based on sphingosine base, was derived from $(E_1 - E_2)$.

The amount of sphingosine (μg . in 10 ml. of *iso*-amylalcohol) = $E \times 250$ (this factor was derived from the experimental results).

RESULTS

Estimation of Free Sphingosine Base—The free sphingosine base obtained from cerebroside was dissolved in benzene and used. The results obtained are shown in Table I.

On the Oxidizing Agent—L. T. A. is easy to be decomposed in the solution, therefore it can not be stored. But, its crystal can be kept in absolute dry condition and used for a long time. It was known that 10 mg. of L. T. A. could oxidise about 1 mg. of shingosine base quantitatively.

Oxidation Time—It was recognized that the suitable oxidation time was 10 minutes as a result of investigations on 5, 10, 20, and 30 minutes.

TABLE I
Photometric Data of the Various Amounts of Sphingosine Base

Experiment	Sphingosine base contained	Extinction
(No.)	(μ g.)	
1	130	0.51
2	130	0.52
3	65	0.26
4	65	0.26
5	32.5	0.13
6	32.5	0.125
7	260	1.03
8	260	1.03

Note: Filter S 55, Cuvette 1 cm., *iso*-Amylalccohol 10 ml.

Estimation of Sphingosine Base in Cerebroside—The hydrolysis of cerebroside was carried out by McKibbin and Taylor's method. The material was hydrolysed with 10 ml. of $\text{Ba}(\text{OH})_2$ by heating in the boiling water bath under reflux for 6 hours and then with 1 ml. of conc. HCl in the same way for 60 minutes. The hydrolysate was made alkaline with NaOH, sphingosine base was extracted with chloroform. This chloroform solution was evaporated, redissolved in benzene and sphingosine base contained was determined as described above.

The results are shown in Table II.

TABLE II
*Analytical Data of Sphingosine Base in Cerebroside after
 Hydrolysis with $\text{Ba}(\text{OH})_2$ and HCl.*

Cerebroside used	Sphingosine base contained in cerebroside	Sphingosine base determined	Recoveries
(mg.)	(mg.)	(mg.)	(%)
1.0	0.362	0.355	98.0
1.0	0.362	0.353	97.4
2.0	0.724	0.682	94.2
2.0	0.724	0.700	96.6

These results show that the hydrolysis with $\text{Ba}(\text{OH})_2$ and HCl, and the extraction with chloroform were satisfactory and the sphingosine base in the hydrolysate of sphingolipids was quantitatively determined.

The effect of cerebron and hydroxy-nervonic acid: These fatty acids, constituents of cerebron and hydroxy-nervon, were also oxidised with L. T. A. and produced higher fatty aldehydes. If they were extracted with chloroform under the conditions described above, the sphingosine base determination would then be affected by their existence. But the results of

the investigation on this problem showed that they did not affect the determination.

The experiment on the recovery of sphingosine base added to cerebroside: The known amounts of cerebroside were mixed with the known amounts of sphingosine base, hydrolysed and free sphingosine base determination was carried out.

The results obtained are shown in Table III.

TABLE III
Recovery of Sphingosine Base Added to Cerebroside

Cerebroside used	Sphingosine base contained in cerebroside	Sphingosine base added	Sphingosine base determined	Recoveries
(mg.)	(mg.)	(mg.)	(mg.)	(%)
1.0	0.362	0.200	0.537	95.5
1.0	0.362	0.200	0.531	94.5
2.0	0.724	0.400	1.091	97.0
2.0	0.724	0.400	1.065	94.0

These results show that the sphingosine base contained in the hydrolysate can quantitatively be recovered and did not change during the processes of hydrolysis.

Estimation of Sphingosine Base in Tissue Lipids—Using the above described method, sphingosine base contents in some tissue lipids were determined.

The results are shown in Table IV.

TABLE IV
Sphingosine Base Content in Human Serum, Brain, Rat Brain and Liver

Tissue	Sphingosine base content (mg./100 g. of fresh tissue)
Human serum	4.4
Human brain	660.0
Rat brain	640.0
Rat liver	11.3

SUMMARY

A colorimetric method of sphingosine base determination was devised. The principle was based on the colorimetric determination of higher fatty aldehyde produced by the oxidation of sphingosine base with lead tetraacetate.

At first the experiment on free sphingosine base was carried out and it was shown that higher fatty aldehyde was quantitatively produced, in proportion to sphingosine base content, by the oxidation with lead tetraacetate. Furthermore, this method was found to be applicable to the determination

of sphingosine base in lipids.

Finally sphingosine base contents in human serum, brain, rat brain and liver were determined.

The author wishes to express his deep indebtedness to Prof. K. Ohno for his helpful criticism on this work.

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PHOTOPHOSPHORYLATION BY CHLOROPLASTS

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The *in vitro* photophosphorylation reaction with preparations from photosynthetic organisms has been the subject of several investigators. Arnon *et al.* (1-5) reported the photosynthetic phosphorylation catalyzed by intact whole chloroplasts and broken chloroplasts. The photophosphorylation by extracts of *Rhodospirillum rubrum* was described by Frenkel (6, 7). Also, the present author (8-10) found that the chloroplast fragments fraction obtained from spinach leaves caused phosphorylation not only by the oxidation of added Krebs cycle intermediates, but also upon illumination without added substrate. The rates of the PSP* reactions with those preparations thus far obtained, however, were too small to explain the large reaction rate of phosphorylation supposed to be functioning in the mechanism *in vivo*. Furthermore, the rates of the PSP reaction with my chloroplast fractions (10) varied from preparation to preparation and the data were often nonreproducible. The present paper deals with the methods of preparation of the chloroplast fractions and the better conditions for the PSP activities which would give higher and reproducible activities of PSP. The results obtained about the properties of the PSP reaction under the improved conditions thus devised are also described. Recently in a preliminary paper (11), Arnon *et al.* reported higher activities of PSP than those previously reported under an improved condition similar to that described in this paper.

MATERIALS AND METHODS

Preparation of Chloroplasts—Unless otherwise stated, the chloroplast fractions were prepared under the following standard conditions. Spinach leaves were washed and removed from midribs. Ten grams of leaves were placed in a chilled porcelain mortar together with coarse emery powder. After the addition of chilled grinding solution (30 ml., containing the following components in final concentrations: sucrose, 0.23 M; ascorbic acid neutralized to pH 7.0 with KOH just before use, 0.02 M; EDTA, 0.002 M and tris buffer of pH 7.8, 0.1 M), the leaves were ground and the mash was strained through a layer of cotton cloth.

* The following abbreviations are used: photosynthetic phosphorylation or photophosphorylation, PSP; oxidative phosphorylation, OXP; ethylene diamine tetraacetic acid, EDTA; tris (hydroxymethyl) aminomethane, tris; adenosine di- or triphosphate, ADP or ATP; flavine mononucleotide, FMN; di- or triphosphopyridine nucleotide, DPN or TPN; 2,4-dinitrophenol, DNP; and inorganic orthophosphate, Pi.

After centrifuging the strained mash for 5 minutes at 1,000 r.p.m. (ca. $100\times g$), the supernatant layer was centrifuged again for 10 minutes at 3,000 r.p.m. (ca. $1,000\times g$). The precipitates thus obtained were suspended with a syringe in 15 ml. of the washing solution (containing the following components in final concentrations: sucrose, 0.23 *M*; potassium ascorbate, 0.02 *M*; EDTA, 0.002 *M*; and tris buffer of pH 7.5, 0.04 *M*). The centrifugation of the suspension was repeated once more as described above. The precipitates were suspended in 10 ml. of the suspending solution (containing the following components in final concentrations: sucrose, 0.18 *M*; potassium ascorbate, 0.05 *M*; EDTA, 0.002 *M*; and tris buffer of pH 7.5, 0.04 *M*). This fraction was designated as the whole chloroplasts fraction (W). For the preparation of the chloroplast fragments fraction (F), the strained mash was centrifuged for 15 minutes at 3,700 r.p.m. (ca. $1,500\times g$), and the supernatant fluid was again centrifuged for 15 minutes at 12,000 r.p.m. (ca. $10,000\times g$). The green precipitates (the supernatant was almost free from greenish tint) were suspended in 15 ml. of the washing solution, and washed by two successive centrifugations, first for 10 minutes at 3,700 r.p.m. and second for 15 minutes at 12,000 r.p.m. The precipitates formed from the first centrifugation were discarded. The green precipitates obtained from the second centrifugation were suspended in 5 ml. of the suspending solution. All the procedures were carried out in a cold room ($1-4^{\circ}$) or in a refrigerated centrifuge.

Preliminary experiments showed that the pH of the ground mash had a marked effect on the activity of chloroplast preparations thus obtained. The optimum pH of the mash was 7.7-7.8. Grinding the leaves at higher or lower pH values always gave chloroplast fragments fraction with lower PSP activities. Therefore, the leaves were always ground in the presence of enough amounts of tris buffer of pH 7.8, namely 3 millimoles per 10 g. of leaves, in order to maintain the pH of the ground mash above 7.6. The effect of the presence of ascorbate during the preparation procedure will be described below. It seemed that grinding leaves with hypertonic solutions gave chloroplast fragments fractions with higher PSP activities, but the effect of tonicity during the preparation was less significant than those of the above two factors. Sucrose could be replaced by either KCl or NaCl without considerable effect. The addition of EDTA to the solutions of preparation was without significant effect.

Measurement of Photophosphorylation—Unless otherwise stated, photophosphorylation was measured in the following way. The reaction mixture, in a total volume of 3 ml., contained the following components in micromoles: sucrose, 300; glucose, 50; tris buffer of pH 8.0, 200; potassium phosphate, 25; $MgCl_2$, 10; ATP or ADP, 3; FMN, 0.3; yeast hexokinase, 200 Kunitz units and the chloroplasts suspension, 0.5-1 ml. Since the chloroplasts suspension contained a sufficient amount of ascorbate, its further addition was unnecessary. Tonicity of the reaction mixture was controlled with the amount of sucrose added, so as to bring the sum of the final concentrations of all the ions and molecules to 0.25-0.35 *M*. The final chlorophyll concentration of the reaction mixture was 0.07 to 0.2 mg. per 3 ml. $MgCl_2$ or tris buffer was added finally to the reaction mixture.

Four chilled Erlenmeyer flasks of 20 ml. capacities, each containing 3 ml. of the reaction mixture, were placed in a water bath of $12\pm 0.5^{\circ}$ and illuminated with a 500-watt spot light at a distance of 50 cm. The light beam was reflected by a mirror placed at the bottom of the bath so that the four vessels were evenly illuminated from the bottom. The light intensity was controlled by a voltage regulator. The voltage was usually set at 85 volt in order that the light intensity at the bottom of each vessel was about 10,000 lux. At intervals, aliquots were withdrawn, deproteinized with 5 per cent perchloric acid, and inorganic phosphate was determined colorimetrically by a modified method of Allen (12). The activity of the photophosphorylation was expressed in micromoles of Pi disappeared during 30 minutes illumination per 0.5 mg. of chlorophyll. Chlorophyll was

determined colorimetrically at 652 m μ by the method of Arnon (13).

Measurement of the activities of oxidative phosphorylation was carried out as described previously (8) except for the following modifications: tris buffer of pH 7.5 was added to the reaction mixture at a final concentration of 0.067 M, final concentration of sucrose was reduced to 0.15 M, NaF was omitted and the temperature of the reaction was 16°.

Materials—ATP and ADP were prepared by means of the anion exchange resin chromatography (14) from crude Ba salt of ATP obtained from rabbit skeletal muscle. The Ba salt of ATP or ADP was decationized by treatment with Amberlite IR-120 and neutralized to pH 7.0 with KOH.

Yeast hexokinase was prepared from baker's yeast according to the method of Slater (15) and was freed of ammonium salts by dialysis and treatment with Amberlite IR-120 resin. The final hexokinase preparation was free from ammonium ion as determined by the Nessler's test and its activity at 15° was 2,000 Kunitz units per ml.

Crystalline bovine serum albumin was kindly supplied from the Armour and Co.

No special attempt was made for obtaining spinach leaves of the uniform quality. They were purchased every morning from the market, care being taken to select apparently healthy leaves.

RESULTS

Determination of the Method of Measurement of the PSP Activities—Reasons for adopting experimental conditions described under the heading "Method" for measurement of PSP activities are as follows.

Time Course of the Photophosphorylation Reaction and Effect of Air—Fig. 1 shows the time course of the photophosphorylation under the standard condition. As can be seen from the figure, the chloroplast fragments fraction consumed inorganic phosphate in the medium under aerobic condition almost linearly with time during the first 30 minutes. The same was true for the whole chloroplasts fraction. The activity of the phosphorylation reaction of the chloroplast fragments fraction was 65 micromoles during the first 30 minutes, and 107 micromoles per 60 minutes. The value for the whole chloroplasts was 77 micromoles per 30 minutes. Both fraction had similar activities on the chlorophyll basis.

One of the large differences between experimental conditions of Arnon *et al.* and those of mine is the necessity of removal of oxygen. Fig. 1 also shows the effect of the replacement of air by nitrogen. Phosphorylation proceeded in nitrogen equally as well as in air during the first 30 minutes, but thereafter its rate in nitrogen was considerably suppressed. Thus, the phosphorylation in nitrogen during the subsequent 30 minutes was only 14 or 19 in contrast to the value of 42 in air. Similar experiments often showed lower activities in nitrogen even during the first 30 minutes, accompanying an earlier decrease in the rate (*cf.* Table VII). Therefore, it may be concluded that the photophosphorylation proceeds quite well in air, and removal of oxygen does not produce any promoting effect.

Effect of Light—As shown in Table I, the phosphorylation reaction was dependent on the light supply. Without light, only a negligibly small phosphorylation occurred, therefore, the contribution of the OXP activity

to the photophosphorylation reaction under the standard condition seemed to be negligible. Under the experimental conditions used, the activity increased in parallel with the increase in the light intensity. At the light intensity of about 10,000 lux, the system seemed to be saturated with light.

Proportionality between the Rate and the Content of Chlorophyll—Fig. 2 shows the results of experiments with varied amounts of the chloroplast fragments

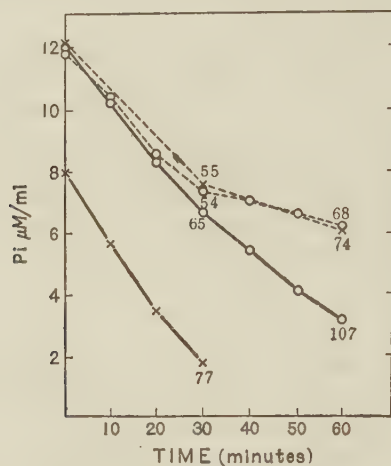


FIG. 1. Time course of the photophosphorylation and effect of anaerobiosis.

Solid line with circles: The chloroplast fragments fraction at a final chlorophyll concentration of 0.041 mg./ml. under aerobic condition. Broken lines with circles or crosses: the same chloroplast fragments under anaerobic condition. Oxygen-free nitrogen was bubbled through each reaction mixture at a rate of 2 liter/hour. These two experiments differed only in numbers of time of sampling, in order to test the effect of air penetrated at the time of sampling. Coincidence of the two broken lines would indicate the absence of such an effect. Solid line with crosses: the whole chloroplasts fraction at a final chlorophyll concentration of 0.040 mg./ml. under aerobic condition. Numerical values in the figure indicate activities, *i.e.*, Pi uptake in micromoles/0.5 mg. chlorophyll. In these experiments, initial concentrations of Pi were increased as illustrated in the figure, in order to maintain the Pi concentrations throughout the experiments sufficient for the phosphorylation reaction.

fraction. The phosphorylation proceeded linearly with time and the rates were in proportion to the chlorophyll contents within a range from 0.02 to 0.06 mg. per ml.

The light-dependency, the linearity of the decrease of Pi during the first 30 minutes and the proportionality of the rates to chlorophyll contents will justify to define the activity of the photophosphorylation reaction in terms of micromoles of Pi disappeared in 30 minutes per unit amount of chlorophyll under the enough supply of light.

Effect of pH—The dependence of the activity upon pH of the reaction mixtures is shown in Table II. Both the chloroplast fragments and whole chloroplasts fractions had their optimum pH at about 8.0. At the pH of

TABLE I
*Effect of Light Intensity on the Activity of the Chloroplast
Fragments Fraction*

The reaction mixture contained the chloroplast fragments fraction in a final chlorophyll concentration of 0.044 mg./ml. Activities were expressed in Pi uptake in micromoles/30 min./0.5 mg. chlorophyll.

Voltage	Intensity of light	Activity
0	—	3
35	—	15
50	—	32
70	—	57
85	ca. 10,000 lux	71
100	—	66

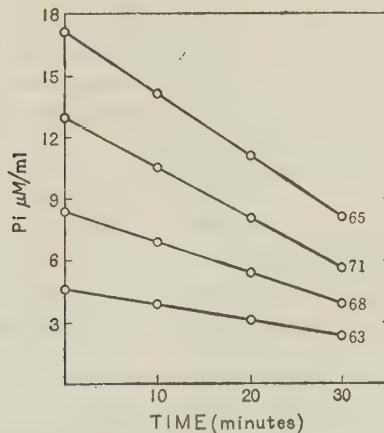


FIG. 2. Proportionality between the phosphate uptake and the concentration of the chloroplast fragments fraction.

From the upper to the lower lines, the reaction mixtures contained chlorophyll in final concentrations of 0.069, 0.051, 0.034 and 0.017 mg./ml., respectively. Initial Pi concentrations were increased as illustrated in the figure from the same reason as described in Fig. 1. The intensity of light was about 10,000 lux. Numerical values in the figure indicate activities, *i.e.*, Pi uptake in micromoles/30 min./0.5 mg. chlorophyll.

7.1–7.3, which was previously used by Arnon *et al.* (4) for their PSP reaction, the activities of both fractions were only half of those at pH 8.0. However, they are now (11) doing their work at pH 8.3, approximately in agreement with the result obtained from this experiment.

Effect of FMN—Table III shows that addition of FMN was necessary for the reaction and confirms the earlier result of Arnon *et al.* (2). At a concentration of 10^{-4} M, the system seemed to be saturated with FMN.

Effect of Tonicity—Effect of tonicity of the reaction mixture on the phosphorylation reaction is shown in Table IV. The activities were not

TABLE II
*Dependence of the PSP Activities of the Chloroplast
Fractions on the pH of the Medium*

pH ^{a)}	Activity ^{b)}	
	F ^{c)}	W ^{d)}
7.1	38	27
7.4	56	45
7.7	63	65
8.0	70 63	63 60
8.3	52	64
8.6	27	31
8.9	18	22

a: Each reaction mixture in a total volume of 3 ml. contained 0.5 ml. of 0.5 M tris buffer of pH as indicated.

b: Pi uptake in micromoles/30 min./0.5 mg. chlorophyll.

c: The chloroplast fragments fraction.

d: The whole chloroplasts fraction.

TABLE III
*Effect of FMN on the PSP Activity of the Chloroplast
Fragments Fraction*

Activities were expressed in Pi uptake in micromoles/30 min./0.5 mg. chlorophyll.

Final concentration of FMN (10^{-5} M)	Activity
0	15
0.4	52
2	65
10	70

significantly affected by tonicities of the reaction mixtures. Another experiments not included in the table showed that sucrose could be replaced by either KCl or NaCl without significant effect.

Effect of Ammonium Ion—Nonreproducible data were often obtained in my earlier experiments. The inhibitory effect of ammonium ion shown in Table V would indicate that one of the causes of this nonreproducibility

would be attributable to ammonium ion remained in the earlier preparation of hexokinase. On the contrary, sulfate ($5 \times 10^{-3} M$ and bicarbonate ($2 \times 10^{-2} M$) ions had little effects on the reaction.

Other Properties of the PSP Reaction—As described above, the earlier procedure of Arnon *et al.* (4) for measurement of the PSP reaction would not give correct figures of the activities. Therefore, the earlier results of Arnon's about the properties of the PSP reaction were re-investigated under improved conditions.

TABLE IV
*Independence of the PSP Activity of the Chloroplast Fragments
Fraction on the Tonicity of the Medium*

Final concentration of sucrose added (M)	Tonicity ^{a)} (M)	Activity ^{b)}
0	0.16	73
0.11	0.27	77
0.21	0.37	76
0.32	0.48	75

a: Tonicity was calculated as the sum of the final concentrations of all ions and molecules present.

b: Pi uptake in micromoles/30 min./0.5 mg. chlorophyll.

TABLE V
*Effect of Ammonium Chloride on the PSP Activity of the
Chloroplast Fragments Fraction*

Activities were expressed in Pi uptake in micromoles/30 min./0.5 mg. chlorophyll.

Final concentration of ammonium chloride added ($10^{-3} M$)	Activity
0	57
0.5	59
1.7	49
5	24

Effect of Ascorbate—In a recent report (11) Arnon *et al.* reported that the presence of 0.01 M of ascorbate during the preparation of their chloroplast preparations protected them from inactivation. (This procedure was already used in my earlier experiment (9)). However, no mention was made about their earlier conclusion that ascorbate is a cofactor of the PSP reaction (2). The effect of addition of ascorbate to the reaction mixture is shown in Fig. 3. In this experiment, the chloroplast fragments fraction was prepared under the condition in which potassium ascorbate was replaced by potassium chloride. Even in the absence of added ascorbate, the phosphorylation still occurred, though the rate of phosphorylation decreased

progressively, the activity per 30 minutes being 50. Since the ascorbate content in the final reaction mixture was found to be less than 0.02 micromole per ml., as determined by the indophenol method, the phosphorylation observed without added ascorbate would not be attributable to such a small amount of ascorbate. On the other hand, the addition of ascorbate to the reaction mixture in a final concentration of $2 \times 10^{-3} M$ did not significantly accelerate the initial rate of the reaction but protected the chloroplasts from later decrease in activity. Consequently, phosphorylation became approximately linear with time and the activity per 30 minutes became

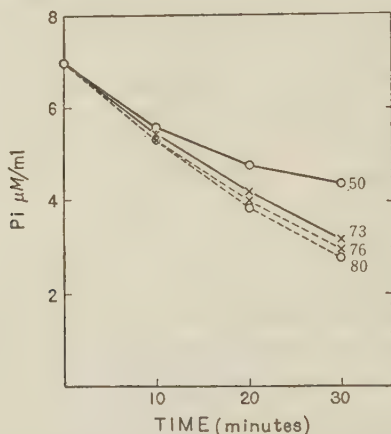


FIG. 3. Effect of ascorbate on the photophosphorylation by the chloroplast fragments fraction.

Solid line with circles: without added ascorbate. The concentration of ascorbate in the reaction mixture was less than $0.02 M$. Solid line with crosses: ascorbate was added in a final concentration of $2 \times 10^{-3} M$. Broken line with circles: ascorbate $7 \times 10^{-3} M$. Broken line with crosses: ascorbate $2 \times 10^{-2} M$. The chloroplast fragments fraction was prepared without added ascorbate. Each reaction mixture contained chlorophyll in a final concentration of 0.026 mg./ml. Numerical values in the figure indicate activities, i.e., P_i uptake in micromoles/30 min./0.5 mg. chlorophyll.

to 73, indicating an apparent accelerating effect of added ascorbate. Further additions of ascorbate were without significant effect. Thus, the effect of addition of ascorbate seemed to be in its protective effect on some of the active centers of the chloroplast preparations rather than its direct participation in the reaction. The effect of the presence of ascorbate during the preparation of the chloroplast fraction (Table VI) would also support the view. The presence of ascorbate during the preparation and the storage protected the chloroplast fraction from inactivation. Particularly during the storage, the more ascorbate was present, the less was inactivation. However, this stabilizing effect of ascorbate was replaced by neither cysteine, gluta-

TABLE VI

Effect of the Presence of Ascorbate during the Course of Preparation of the Chloroplast Fragments Fraction

The PSP activities were expressed in Pi uptake in micromoles/30 min./0.5 mg. chlorophyll.

Exp. No.	Concentration of ascorbate during the PSP reaction ($10^{-3} M$)		Activity	Concentration of ascorbate during the storage ($10^{-3} M$)	Activity after storage for 3 hrs. at 0°
1 ^a)	0		48	0	28 ^b)
	2.4		59	20	41
	8		63	45	48
	24		63	100	57
2 ^c)	Concentration of ascorbate ($10^{-3} M$)				
	At the time of grinding	During the PSP reaction			
	0	0	27	0	3 ^d)
	4	1	45	5	20
	15	4	53	20	21
	38	10	52	50	32

a: Leaves were ground in the presence of ascorbate and the chloroplast fragments fraction was washed and suspended in ascorbate-free medium. PSP activities were measured with various additions of ascorbate. Another aliquots of the suspension were added with various amounts of ascorbate and stored in the dark for 3 hours at 0° , and then their PSP activities were again measured.

b: Appropriate amount of ascorbate was added to the reaction mixture to give a final concentration of $5 \times 10^{-3} M$.

c: From four batches of leaves, each weighing 10 g., the chloroplast fragments fractions were prepared with various amounts of added ascorbate, and activities were measured. Each chloroplast suspension was stored in the dark for 3 hours at 0° , and then activities were again measured.

d: No ascorbate was added to the reaction mixture.

thione, bovine serum albumin nor egg albumin. It was also observed that, once the activity was decreased, a renewed addition of ascorbate did not restore the original activity.

Effect of Menadione—Although the results of Arnou *et al.* (3) showed the accelerating effect of added menadione on their PSP reaction disregarding the presence of FMN, no such accelerating effect of menadione could be found in my earlier experiment (10). My experimental condition was aerobic, whereas they stated that the effect of added menadione was only remarkable under anaerobic condition. Therefore, the effects of menadione under both aerobic and anaerobic conditions were re-investigated with my improved procedure. A representative data is shown in Table VII. Addition

of menadione to 10^{-3} *M* did not significantly accelerate the reaction both under aerobic and anaerobic conditions. Another experiments with 10^{-5} *M* menadione or 10^{-4} *M* menadione phosphate gave similar results. None of the experiments showed higher activities under anaerobic condition with added menadione. Thus, addition of menadione was not necessary if enough

TABLE VII
Effect of Menadione on the PSP Activities of the Chloroplast Fractions

Final concentration of menadione (10^{-3} <i>M</i>)	Gas phase	Activity ^{a)}	
		F ^{b)}	W ^{c)}
0	air	53	53
0.1	air	52	53
0	nitrogen ^{d)}	23	26
0.1	nitrogen ^{d)}	32	25

a: Pi uptake in micromoles/30 min./0.5 mg. chlorophyll.

b: The chloroplast fragments fraction.

c: The whole chloroplasts fraction.

d: Oxygen-free nitrogen was bubbled through the reaction mixture at a rate of 1 liter/30 min.

TABLE VIII
Effects of DNP on the PSP and OXP Activities of the Chloroplast Fractions

DNP was added just before the start of illumination.

Final concentration of DNP (<i>M</i>)	PSP activity ^{a)}		OXP activity ^{a)} of F ^{b)}
	F ^{b)}	W ^{c)}	
0	69	63	3.8
3.3×10^{-5}	68	60	—
1×10^{-4}	—	—	-0.1 ^{d)}
2×10^{-4}	62	59	—
1×10^{-3}	29	27	—

a: Pi uptake in micromoles/30 min./0.5 mg. chlorophyll.

b: The chloroplast fragments fraction.

c: The whole chloroplasts fraction.

d: The concentration of Pi increased slightly.

FMN was present. However, the possibility still remained that the effect of added FMN (Table III) could be replaced by menadione in the absence of FMN.

Effect of DNP—In an earlier paper (4) Arnon *et al.* reported that their PSP reaction with whole chloroplasts was inhibited with 8×10^{-4} *M* DNP by

more than 80 per cent whereas Frenkel's photophosphorylation system was not significantly affected with 10^{-4} M DNP (6). Table VIII shows the effects of addition of DNP on the PSP activities of the two chloroplast fractions under my improved conditions. DNP at 2×10^{-4} M did not significantly inhibit the PSP reactions of the two fractions. Only at 10^{-3} M, DNP inhibited the PSP reactions by approximately 60 per cent. These results are consistent with the results of the two above-mentioned experiments and are in contrast with the result that the OXP reaction by the same chloroplast fragments fraction was inhibited almost completely with 10^{-4} M DNP.

DISCUSSION

Arnon (4, 16, 17) criticized my earlier results (8, 9) that my chloroplast fragments might be composed of a mixture of chloroplast fragments and other cytoplasmic particles, presumably mitochondria, and concluded that the activity of PSP was exclusively localized in whole chloroplasts or chloroplast fragments derived from the former. However, his results (*cf.* Table IV in ref. (4)), which he presented as one of the main evidences of the above conclusion, was merely a repetition of my earlier experiment (9), except that his remaining particles had much lower activity of PSP than his whole chloroplasts or my chloroplast fragments fraction. Although the remaining particles were considered to be mainly composed of mitochondria and the content of chloroplast fragments would be less than my chloroplast fragments fraction, the PSP activity of the remaining particles would be attributable to the chlorophyll-containing particles. The lower activity of PSP per unit amount of chlorophyll of the fraction than those of whole chloroplasts or broken chloroplasts would indicate that the PSP activities of chloroplast fragments were more readily destroyed than those of whole chloroplasts or broken chloroplasts, or indicate that the figures of PSP activities which they obtained were incorrect because of their unsuitable procedure of measuring the PSP activity. The same was true for their P_2 fraction (4) which they considered to be composed of chloroplast fragments and other cytoplasmic particles. If these were not the case, the results are nothing but an evidence that the remaining particles or P_2 fraction contained the third chlorophyll-containing particles with much lower PSP activity per unit amount of chlorophyll than whole chloroplasts or broken chloroplasts.

The activity of PSP of my chloroplast fragments fraction was high enough to correspond to that of whole chloroplasts, in contrast to those of the remaining particles and P_2 fraction of Arnon *et al.* (4). However, the result only indicates that both the whole chloroplasts and the chloroplast fragments fractions showed similar PSP activities per unit amount of chlorophyll and does not answer the question whether or not the chloroplast fragments fraction contained small non-chloroplast particles, presumably mitochondria. At present I have as yet no answer, except that all the particles with OXP activities thus far obtained from green leaves were green. However, I find

an indication in a recent paper of Arnon *et al.* (11) that the chlorophyll-containing particles in my chloroplast fragments fraction might be different from the broken chloroplasts obtained from whole chloroplasts by breaking them with hypotonic treatment. That is the fact that my chloroplast fragments fraction had similar activities of PSP without added TPN or chloroplast extract (50–75 micromoles of Pi per 0.5 mg. of chlorophyll per 30 minutes) as those of broken chloroplasts with added TPN and chloroplast extract (4.3 and 6.7 micromoles of Pi per 0.1 mg. of chlorophyll per 20 minutes). Thus, my chloroplast fragments fraction seemed to contain enough TPN and chloroplast extract and not to have been subjected to such treatment as hypotonicity which lead them to lose their components.

The fact that the PSP activities of the chloroplast preparations became higher as the preparative procedures were improved suggests that the improved procedures protected the PSP activity of chloroplasts from inactivation during the preparation of the *in vitro* system. The lability of the OXP activity of the chloroplast fragments fraction was already described (8). Therefore, I consider that it is meaningless to compare *in vitro* activities of each fraction without evidence that there was no loss of activity during the course of preparations of the systems. Recently, Arnon *et al.* (11) described that the rate of PSP of their most active preparation was 400 micromoles of Pi per mg. of chlorophyll per hour, and stated that the rate was high enough to correspond to that of photosynthesis *in vivo*, namely 200 micromoles of oxygen per mg. of chlorophyll per hour. The rate is much higher than those thus far reported and is still higher than those obtained in the present experiment. However, I consider that still higher rates of PSP would be obtained *in vitro*, because, if 3 moles of ATP were required per mole of oxygen produced in the photosynthesis (18), the rate of phosphorylation *in vivo* should be $3 \times 200 = 600$ micromoles of Pi per mg. of chlorophyll per hour.

If the problems were confined, apart from the localization of the two phosphorylation reactions, to the earlier suggestion of Vishniac and Ochoa (19) that PSP is caused by the coupling of the Hill reaction of chloroplasts with OXP of mitochondria, even the activities thus far obtained may be useful in elucidating this point. Comparison of the two phosphorylation reactions will present evidences for or against the suggestion. The earlier result of Arnon *et al.* (5) that broken chloroplasts which were considered to be inactive in OXP were active in PSP and their newer result (11) that PSP by broken chloroplasts was specifically enhanced by addition of TPN and not by DPN may be strong proofs against the above suggestion. Also much higher activity of PSP than that of OXP of my chloroplast fragments fraction made difficult to explain the PSP reaction with the above mentioned coupling mechanism (9, see also Table VIII). The result obtained in the present paper that PSP was not significantly inhibited by 10^{-4} M DNP which inhibited almost completely the OXP reaction of the same chloroplast fragments fraction would add further evidence against the suggestion. The

PSP reaction in the presence of $2 \times 10^{-4} M$ of DNP would not be explained by the coupling mechanism. Therefore, it is more natural to consider that the PSP reaction is another phosphorylation reaction distinct from the OXP reaction of mitochondria.

SUMMARY

Both the chloroplast fragments and whole chloroplasts fractions had the activities of photosynthetic phosphorylation. The activities of the two fractions were almost equal when calculated on unit amount of chlorophyll. Neither removal of oxygen nor addition of menadione did not produce any accelerating effect on the reaction. From the effect of DNP, it was concluded that the photophosphorylation was a distinct phosphorylation reaction different from oxidative phosphorylation of mitochondria.

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AN APPARATUS FOR STUDY OF THIXOTROPY OF BIOCOLLOID

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Although it has been suggested by several physiologists (1) that the thixotropic property of fibrous protein plays an important role in living cell, progress in elucidation of its molecular mechanism has not yet been greatly made. It is desirable to have a study, in which flow birefringence is measured simultaneously with viscosity. There are two kinds of apparatus, used for the measurement of flow birefringence, namely, the cylinder and the capillary types (2). The former is more frequently used. But Conner and Donnelly (3) recently accomplished, using the latter type of apparatus, the direct measurement of relaxation of birefringence in a concentrated viscose solution under various velocity gradients, and they made great progress in the elucidation of the physicochemical properties of thixotropy.

The apparatus described in this paper was constructed for the study of thixotropy of myosin B, a structural protein from muscle, under the same principle as that of Conner and Donnelly with several improvements. This instrument can also be used for the study on thixotropy of other biocolloidal solutions.

APPARATUS

I. Principle

The velocity gradient is achieved by allowing the solution of highpolymer to flow under pressure head through a cylindrical glass tube which is fixed between two crossed Nicol prisms. A light beam passing through the glass tube is received with a photomultiplier and the relative birefringence Δn_0 is calculated by the relation:

$$\Delta n_0 = K(I_f - I_0) \quad (1)$$

where I_f and I_0 are out-put currents of the photomultiplier when the solution is forced to flow and when the flow is stopped, respectively, and K is an apparatus constant. The decay of out-put current after the stop of flow is recorded on an electromagnetic oscillogram, and the relaxation time of birefringence is read directly from the record. When the Benoit (4) equation

$$\Delta n = \Delta n_0 e^{-\tau/\theta} \quad (2)$$

is satisfied, the relation between the relaxation time (τ) and the rotary diffusion constant

(Θ) is given by the equation:

$$\Theta = \frac{1}{6\tau} \quad (3)$$

where Δn is the relative birefringence at t seconds after the stop of the flow. The length $2a$ of the equivalent elongated ellipsoid and the cylinder is given, respectively, by Perrin's (5) equation:

$$\Theta = \frac{3kT}{16\eta_0 a^3} \left(-1 + 2 \ln \frac{2a}{b} \right) \quad (4)$$

and Burger's (6) equation:

$$\Theta = \frac{3kT}{8\pi\eta_0 a^3} \ln \left(\frac{2a}{b} - 0.80 \right) \quad (5)$$

where b is the semiminor axis and η_0 is the viscosity of the solvent.

A mean value of the velocity gradient ($\bar{\beta}$) in a Newtonian flow is given by the formula of Kroepelin (7):

$$\bar{\beta} = \frac{8V}{3\pi r^3 t} \quad (6)$$

where V is the volume of the solution flowed in time t through the sample cell of radius r . The relative viscosity (η_{rel}) of a polymer solution is obtained as ratio of its flowing time (t) to that of the solvent (t_v) at a constant pressure head:

$$\eta_{rel} = (t/t_v)_p \quad (7)$$

II. Optical System

The optical and mechanical systems are diagrammatically illustrated in Fig. 1. The

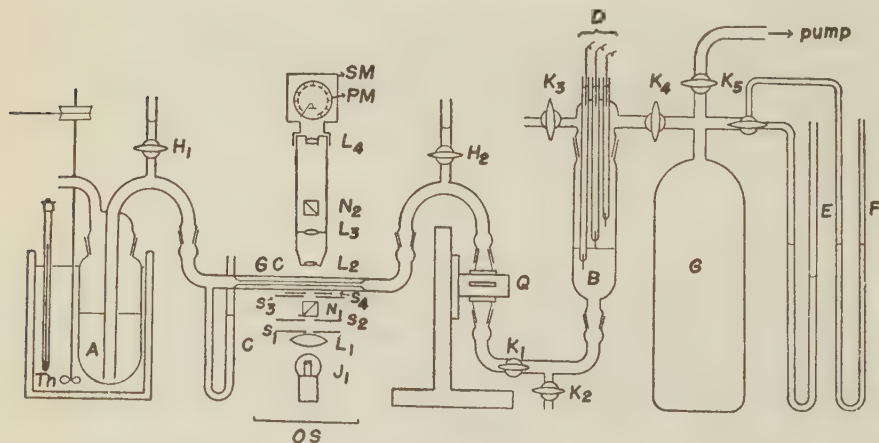


FIG. 1. Apparatus for measurement of flow birefringence and its relaxation. A, reservoir of solution; B, flow meter; D, electrodes of flow meter; E and F, mercuric and water manometer, respectively; GC, sample cell; G, gas reservoir; H_1 and H_2 , cocks to eliminate foam in solution; K_1 , K_2 , K_3 , K_4 and K_5 , stopcocks; OS, optical system; J_1 , light source, Leitz ATIIH (50 candle); L_1 , condenser lens; L_2 , L_3 and L_4 , lenses; S_1 , S_3 and S_3 , iris stops; S_4 , rectangular aperture.

optical system (OS in the figure) was a Leitz universal microscope, Panphot. The light source, J_1 , was a 50 candlepower Leitz special lamp (exclusive use for Panphot, ATIIH) powered by a 6-volt storage battery. Adjustment of distance between the light source J_1 and the condenser lens L_1 is made to produce approximately parallel beam of light passing through the sample cell. Lens system L_2 - L_3 - L_4 was focussed on the sample cell GC which was fixed horizontally on the stage of the microscope. The knife edge slit S_4 was placed just under the sample cell, so that the light beam takes a slender rectangular form along the axis and passes through the central part of the sample cell. The other lenses between L_1 and S_4 were taken off to eliminate the diffused reflection at their surfaces.

The Nicol prisms were crossed with their axes 45° to the axis of the flow tube. The sample cell GC was a thick glass tube of medium quality. To avoid dichroism caused by distortion of glass, annealing of glass tube must be performed with sufficient time, because the genuine value of Δn_0 is seriously damaged by dichroism of glass tube.

III. Mechanical System

The pressure head to flow the solution through the glass tube was achieved, by means of a vacuum pump, by depressing pressure of a gas reservoir of 10 l. in volume. The pressure difference was measured by a mercuric (E) and a water (F) manometer with the aid of a cathetometer, the difference between heights of the surfaces of the solution in the reservoir (A) and the flow meter (B) being compensated. At first, the cock (K_1) was closed and the spring-loaded knife valve (Q) was opened. Then the solution was forced to flow by opening of K_1 . After the flow was stopped by the action of Q, the pressure difference was measured. The solution collected in B was excreted downward by closing the cock K_4 and opening the cocks K_2 and K_3 . The cocks H_1 and H_2 were inserted to eliminate foam from the solution.

The sample cell was 0.12 cm. in inside radius and 38.5 cm. in length. As well known, the flow is laminar below ca. 2,400 of the Reynolds number (θ);

$$R = \frac{2\rho V}{\pi \eta r t} \leq 2,400 \quad (8)$$

where ρ is the density of the solution. Inserting eq. (6),

$$\frac{3\rho\bar{\beta}r^2}{4\eta} \leq 2,400.$$

When $\rho=1$ and $\eta=0.04$ (these values correspond to 0.6 M KCl solution of 6 mg. of myosin B/ml.), the velocity gradient must be lower than $10,000 \text{ sec}^{-1}$ and when $\rho=1$ and $\eta=0.015$ (1.5 mg. of myosin B/ml.), it must be lower than $4,000 \text{ sec}^{-1}$.

Three platinum needle electrodes (D)* were fixed at appropriate intervals in a cylindrical glass vessel (B), 25 cm. in length and 3 cm. in diameter. These electrodes were connected as illustrated in Fig. 2. The condensers C_1 and C_2 were charged when the solution contacted only with the electrode D_1 . After the switch and cock K_1 were opened, the solution began to flow into B. When the circuit C_1 - D_1 - D_2 was closed by contact of the surface of the solution with the electrode D_2 , the vibrator Vb of an electromagnetic oscillograph followed the discharge of C_1 . When the surface reached to D_3 and the circuit C_2 - D_2 - D_3 was closed, Vb vibrated again. The duration of the discharging and the amplitude of the vibration were controlled by the adjustment of resistances of R_1 and R_2 .

* D_1 might be fixed on the vessel wall, but D_2 and D_3 could not be fixed on the vessel wall, because surface membrane of protein on the wall caused leaking of charges of the condensers.

This flow meter can be, of course, used only for electrolytic solution. A type D vibrator was used for 0.6 *M* KCl solution, since in this case the resistances between D_1 and D_2 and between D_2 and D_3 were 500 and 200 kohm, respectively. Fig. 3 represents an example of the record. In the present apparatus the volume between the tops of the two electrodes D_2 and D_3 was rather large (17.02 ± 0.02 ml.). But, a thinner vessel and a shorter distance between D_2 and D_3 must be used for measurements of those biocolloids difficult to obtain in large quantity.

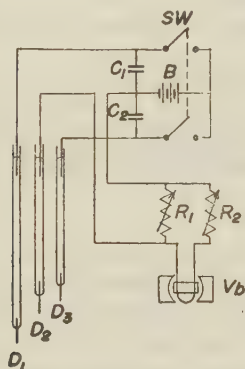


FIG. 2. Electrical circuit for measuring flow time. D_1 , D_2 and D_3 , platinum electrodes; B, battery, 7.5 V; C_1 and C_2 , condensers, 0.2 μ F; Vb, vibrator of electromagnetic oscillograph, D type; Sw, switch.

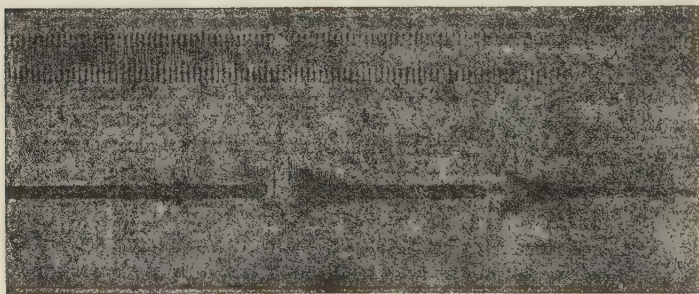


FIG. 3. Electromagnetic oscillogram for measuring flow time. 5.94 mg. of myosin B/ml, 0.55 *M* KCl, pH 6.2-6.3, 20°. Pressure head, 390 cm. H_2O .

To measure relaxation of birefringence, the flow must be stopped as quickly as possible without a violent turbulence of the solution. A spring-loaded knife-edge valve (Fig. 4) of stainless steel was constructed for this purpose. Two pin holes (PH₁ and PH₂) were bored in the sliding parts (SL), so that the light beam from the light source (J_2) was able to reach a phototube (PT) only through the two pin holes when SL moved. The shutter speed could be measured by recording two flicking lights received by PT. As shown in Fig. 5, the speed of sliding of SL was 1/7 cm. msec⁻¹. When it is assumed that the Poiseuille law can be adopted by equilibration the flow near the shutter hole to the one through a cylinder of the same area and 0.1 cm. or 0.5 cm. in length, the time interval necessary to stop the flow could be calculated to be 0.01 msec. or 0.03 msec. Of course, the flow near the shutter hole could not be laminar, but as the edge of the valve was rather keen, the values may be rather over-estimated. Therefore, a relaxation longer than about 0.2 msec. may be measured by the present spring-loaded knife valve. When a more viscous solvent and a longer and finer capillary were used, rotation of a particle shorter

than 2,800 Å can be measured. For instance, when the viscosity of solvent is 0.04 and the sample cell is a capillary 0.5 cm. in inside radius and 50 cm. in length, the stop of flow may be sufficiently shorter than rotation of elongated particle of 1,000 Å in length.

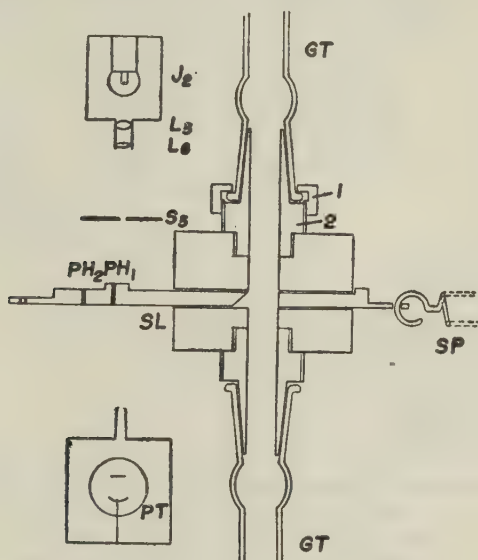


FIG. 4. Spring-loaded knife valve. SL, sliding part; SP, spring; GT, glass tube; J₂, light source (automobile lamp); L₅ and L₆, lenses; PH₁ and PH₂, pin holes; PT, phototube.

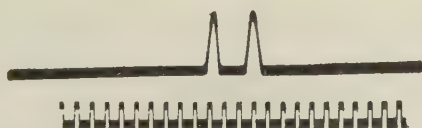


FIG. 5. Sliding speed of SL of knife valve. Time scale, 400 c/s.

IV. Electrical System

A wiring diagram of the voltstat power supply for the photomultiplier RCA 1P21 is illustrated in Fig. 6. The voltage between the cathode and the 9th dinode of the photomultiplier was about 600–800 v. When a higher voltage was demanded, fifteen dry batteries BL 145 (67.5 v) were used as the power supply, since this voltage controlling circuit began to oscillate. A metal shielding house SM, enclosing the valve, was connected to earth to minimize noisy current. As shown in Fig. 7, the tube was also enclosed by a glass vessel (GM) and dried nitrogen gas was passed through the vessel. And yet, noise in the output current from the photomultiplier limited the accuracy of measurement. It is expected that such a noise can be eliminated by lowering the temperature of PM by means of liquid air.

As the relaxation of birefringence of myosin B solution was about 10 msec., it was recorded directly on an electromagnetic oscillogram with the aid of a type H vibrator. This method is superior to that of Conner and Donnelly in such a point that it can

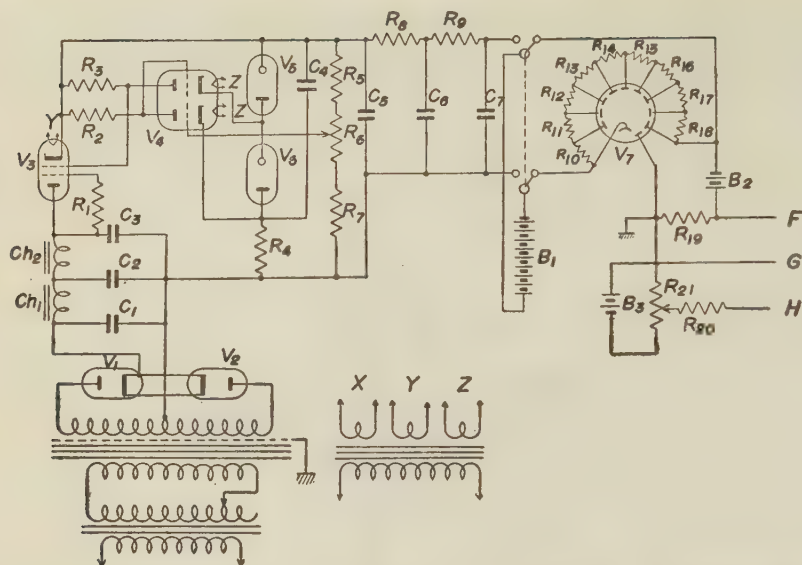


FIG. 6. Wiring diagram of power supply for photomultiplier. V_1 and V_2 , HX 968 (Toshiba); V_3 , 807; V_4 , 12AX7; V_5 and V_6 , VR150; V_7 , 1P21 (RCA). Ch_1 and Ch_2 , 20 H. B_1 , 67.5 v $\times$ 15; B_2 , 90 v; B_3 , 6v. R_1 , 100 ohm; R_2 , and R_3 , 500 kohm; R_4 , 30 kohm 25 w; R_5 , 500 kohm; R_6 , 1 Mohm; R_7 , 500 kohm; R_8 and R_9 , 20 kohm; R_{10} - R_{18} , 500 kohm; R_{19} , 2 Mohm (grid leak of amplifier); R_{20} , 2 Mohm; R_{21} , 50 kohm. C_1 - C_3 , 8 μ F, 2000 wv; C_4 , 0.1 μ F; C_5 - C_7 , 10 μ F, 2000 wv. F, G and H are connected with F, G and H of Fig. 8, respectively.

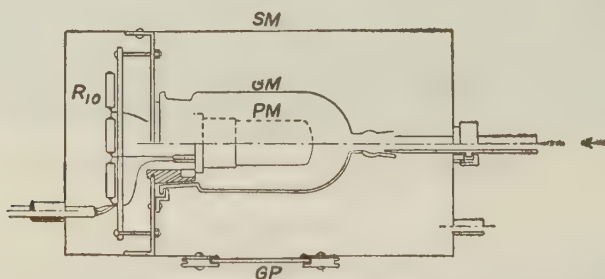


FIG. 7. Shielding house of photomultiplier. SM, metal shielding house; GM, glass vessel; GP, glass plate; R_{10} , resistors.

record a relaxation process just as it stands. Since current necessary for the full scale vibration of a type H vibrator is about 5 mA, a direct current amplifier (Fig. 8) was built for this purpose, using a power amplifying pentode 6BQ5 (Matsushita Elec. Co.). The driving voltage of this tube is relatively low, and 2 Mohm was used as the grid leak of this tube, and yet, the current ($I_f - I_0$) was so small that preamplifier employing a tube 12AT7 (Fig. 9) was necessary in order to measure low intensity of flow birefringence.

Fig. 10 is an example of a record of the relaxation of birefringence of myosin B. Current of the pulse oscillator (400 c/s) was recorded as a time scale with the aid of a type A vibrator.

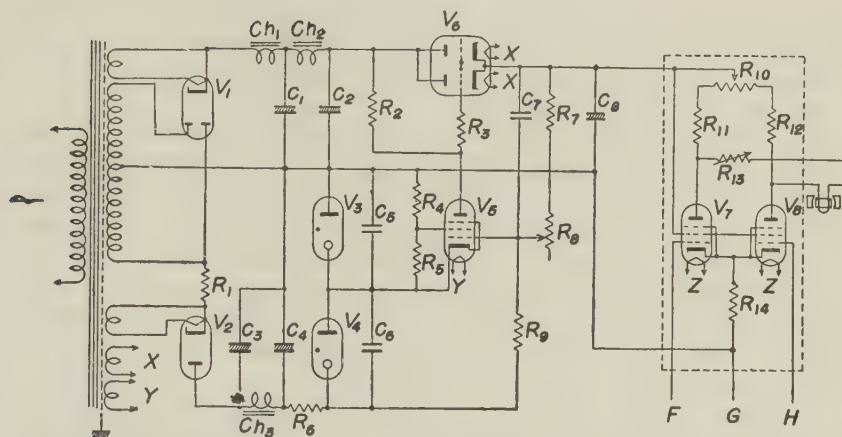


FIG. 8. Electric circuit of amplifier. V_1 , 5AR4; V_2 , 12FK; V_3 and V_4 , VR150; V_5 , 6SJ7; V_6 , 6AS7; V_7 and V_8 , 6BQ5. C_1 and C_2 , $40\ \mu\text{F}$; C_3 and C_4 , $20\ \mu\text{F}$; C_5 and C_6 , $0.01\ \mu\text{F}$; C_7 , $0.1\ \mu\text{F}$; C_8 , $40\ \mu\text{F}$. Ch_1 and Ch_2 , 10 H, 250 mA; Ch_3 , 30 H, 50 mA. R_1 , 1 kohm, 2 w; R_2 , 1 Mohm; R_3 , 1 kohm; R_4 and R_5 , 30 kohm; R_6 , 3 kohm 5 w; R_7 , 200 kohm; R_8 , 1 Mohm; R_9 , 250 Mohm; R_{10} , 0.2 Mohm, 5 w; R_{11} and R_{12} , 1 kohm, 10 w; R_{13} , 0.6 kohm, 2 w; R_{14} , 85 Ohm, 10 w.

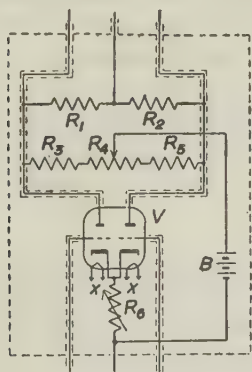


FIG. 9. Wiring diagram of preamplifier. V , 12AT7; B , battery, 67.5 V; X , connected to storage battery (6V); R_1 and R_2 , 1 Mohm; R_3 and R_5 , 10 kohm; R_4 , 5 kohm. R_6 , 300–600 ohm. F_1 , G_1 and H_1 are connected to F , G and H of Fig. 6, F_2 , G_2 and H_2 to F , G and H of Fig. 8, respectively.

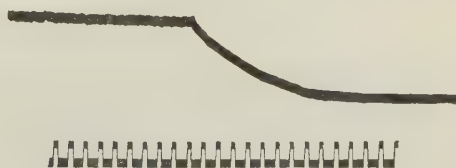


FIG. 10. Record of relaxation of birefringence of myosin B. 2.34 M KCl, pH 6.7, 20° , myosin B 3 mg./ml., velocity gradient $\sim 5,000\ \text{sec}^{-1}$. Time scale, 400 c/s.

DISCUSSION

The special feature of this apparatus is that the dependence of intensity of flow birefringence, viscosity and rotary diffusion constant on the velocity gradient (β) can be measured, and especially that relaxation of birefringence

can be directly observed under various velocity gradients. This is a big advantage for the study of thixotropy of polymer solution. The details of the results on myosin B solution obtained with the present apparatus will be described in the other paper (9).

The present apparatus, however, has also some serious faults. First, the relaxation time must be longer than 0.1 msec. Second, velocity gradient is given only as a mean value, and the range of β where the flow is laminar, is not very wide. Third, the greatest fault for the study on a biocolloid is that a considerable amount of any sample solution is needed. However, these faults except the one that β is given only as a mean, can be overcome by improvements of the apparatus, especially in amplifier and recorder. At present, for the study of thixotropy, the measurement of dependency of viscosity and relaxation time of birefringence on velocity gradient is the most excellent method. Therefore, it is expected that pertinent information on thixotropy of solutions of biocolloids such as gelation, tobacco mosaic virus, F-actin and nucleic acid may be obtained with the aid of this kind of apparatus.

SUMMARY

An apparatus for measurements of the dependency of viscosity, intensity of flow birefringence and relaxation of birefringence on the velocity gradient was constructed, employing a sample cell of a capillary type. With this apparatus, relaxation longer than 0.2 msec could be recorded.

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THE STRUCTURE OF CYTOCHROME C

II. PROPERTIES OF ACETYLATED CYTOCHROME C

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Over the past several decades, many investigators have shown their interests in the contribution of the protein structure to the function of enzymes. Studying the chemical structure of cytochrome c (1), the authors have tried to elucidate the role of the protein structure on the catalytic activity of the enzyme.

The questions are how and what part of the protein moiety plays the role in catalytic function of cytochrome c. The question may be partly solved by studying catalytic and other properties of cytochrome c with its functional groups substituted. As cytochrome c is a remarkably basic protein and the content of lysine amounts to 20 per cent of amino acids residues, the blocking of amino groups was undertaken at first.

In the present article, cytochrome c was acetylated by treating with acetic anhydride and changes of its catalytic activities as well as other characteristic properties were examined.

EXPERIMENTAL AND RESULTS

Cytochrome c—Cytochrome c was extracted from horse heart muscle with sulfuric acid. The extract was neutralized with sodium hydroxide and diluted to a favourable cationic concentration (0.1 Na⁺g ions/liter). The pigment was absorbed on ion exchange resin Amberlite IRC-50 and eluted with 10 per cent ammonium sulfate solution. The eluate was dialyzed and chromatographed over the resin according to the method of Boardman and Partridge (2). The eluate was again dialyzed and absorbed on the resin. The eluate with ammonium hydroxide was lyophilized.

Preparation of Acetyl-derivative of Cytochrome c—Cytochrome c in half saturated sodium acetate solution was treated with acetic anhydride in an ice bath. According to Fraenkel-Conrat and Olcott (3), this technique was found to cause acetylation of only the amino groups and the free sulphydryl groups of proteins. Appropriate amounts of acetic anhydride (4 to 200 moles of the reagent per mole of the protein) were added with stirring at 0–5° to the solution of cytochrome c. After half an hour, the

reaction mixture was divided into three portions. The first portion was diluted with 0.1 *M* phosphate buffer, pH 7.0, and used for the spectrophotometric study. The second portion was dinitrophenylated by the method of Sanger (4) for the estimation of residual amino groups. The third portion was dialyzed successively against running water and 0.1 *M* phosphate buffer and then used for the studies of catalytic and other properties.

Estimation of the Number of Acetylated Amino Groups—The acetylated cytochrome *c* was analyzed for amino groups remaining by the DNP-method. After hydrolysis of the DNP-acetylated cytochrome *c* with 6 *N* HCl for 10 hours in boiling water bath, dinitrophenylated amino groups were estimated spectrophotometrically at 400 μ m in acidic medium to avoid the absorption based on dinitrophenol. As the standard, a valine solution of known concentration was treated in the same way and estimated at the same time. The estimation of the number of acetylated amino groups was made by assuming the number of amino groups in the original cytochrome *c* to be 20. (The number of lysine residues in horse cytochrome *c* molecule is about 18 by DNP-method (5). Cytochrome *c* was claimed to have 2 α -amino terminal residues according to Margoliash (6).)

By the above procedure were obtained cytochrome *c* derivatives acetylated to various degrees, up to 90 per cent amino groups.

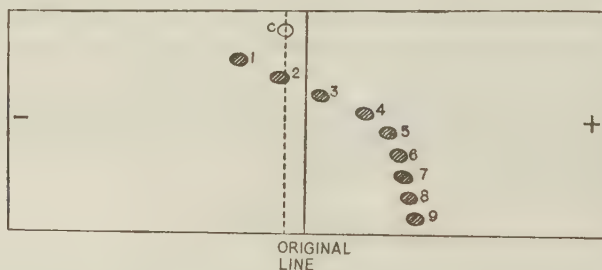


FIG. 1. Paper electrophoretic pattern of acetylated cytochrome *c*.

Paper electrophoresis was followed under the following condition: 0.25 mA per cm. width, 10 volts per cm. length, 5 hours, 5–10°. Buffer was sodium veronal-hydrochloric acid, $\mu=0.1$, pH 8.4. c: glucose, 1: the original horse cytochrome *c*, 2: 17 per cent acetylated, 3: 44 per cent acetylated, 4: 68 per cent acetylated, 5: 75 per cent acetylated, 6: 84 per cent acetylated, 8: 85 per cent acetylated, 8: 87 per cent acetylated, 9: 89 per cent acetylated.

Paper Electrophoresis—Paper electrophoresis was used to show the homogeneity and the change of isoelectric point of acetyl derivative of cytochrome *c*. Toyo filter paper No. 51 was used and a potential of 7 to 10 volts per cm. length was applied to give a 0.25 mA per cm. width. Buffer used was sodium barbiturate-hydrochloric acid (pH 8.4, $\mu=0.1$). Glucose was used as a control to show the extent of electroosmosis.

Fig. 1 shows the paper electrophoretic diagram of cytochrome *c* acetylated

to different extents. The acetylated derivative was homogeneous at least electrophoretically. It moved to cathode at pH 8.4, whereas the native or the slightly modified one moved to anode at this pH. Electrophoretic mobility to cathode increased as the acetylation proceeded.

Ion Exchange Resin Chromatography—The acetylated cytochrome c was examined for the chromatographic behaviour using Amberlite IRC-50 (200 to 400 mesh) column (0.9×10 cm.) equilibrated with phosphate buffer (0.25 g ion/liter). For the elution was used the same buffer (7).

Cytochrome c acetylated to a considerable extent, 40 per cent of amino groups, could not be absorbed on the above column. However, slightly acetylated one (17 per cent) could be absorbed on the column and when eluted with buffer, movement of the band was rather slow compared to the original one.

Spectrophotometric Study—The solution of acetylated cytochrome c which was diluted with 0.1 M phosphate buffer, pH 7.0, was reduced by addition of sodium dithionite and the intensity of the absorption at $550 m\mu$ was measured. This intensity was calculated by assuming the absorption at $550 m\mu$ of the native cytochrome c of the same concentration as the acetylated one to be 100. For the determination of the affinity towards carbon monoxide, a current of pure carbon monoxide gas was passed for 5 minutes through the solution of cytochrome c in the reduced state and the intensity of the absorption at $550 m\mu$ was measured. The determination was absorption at $550 m\mu$ was measured. The determination was made by the method of Tsou (8). The autoxidizability was estimated from the decrease in the absorption at $550 m\mu$ by aeration. In this case, the reduction of cytochrome c was made with hydrogen and palladium black.

No change in the absorption spectra of both oxidized and reduced form of cytochrome c was observed in the course of acetylation. The fall of the absorption at $550 m\mu$ of ferrocytochrome c was not observed even after 90 per cent of its amino groups were acetylated as shown in Fig. 2. The affinity towards carbon monoxide was examined to detect the existence of the so-called "autoxidizable" fraction of Tsou. The affinity was not observed even after nearly complete acetylation. However, the autoxidizability increased more and more as the substitution proceeded. Finally, the autoxidation of cytochrome c, acetylated more than half of amino groups, was so intensive that the derivative was instantly oxidized completely if reduced by palladium black and hydrogen.

Measurement of Catalytic Activity—The activity of acetyl-derivative of cytochrome c in biological electron transfer was measured using succinic oxidase system of rat liver. The derivative (not more than 3×10^{-8} mole) was added to a succinic oxidase system of rat liver homogenate and oxygen uptake was measured manometrically at 37° (9). An almost linear proportionality was observed between oxygen uptake and the amount of cytochrome c added, when the amount of cytochrome c did not exceed 3×10^{-8} mole.

As shown in Fig 2, the activity of acetylated cytochrome c was decreased in accordance with the progress of the acetylation. Cytochrome c acetylated to a considerable extent (more than half of amino groups) had no activity in succinic oxidase system so far as the present manometric method was concerned.

Enzymatic reducibility of acetylated cytochrome c was estimated spectrophotometrically using a succinic oxidase system of rat liver. The reaction mixture was as follows: 0.016 *M* phosphate buffer, pH 7.4, 0.22×10^{-3} *M* potassium cyanide, 0.40×10^{-3} *M* calcium chloride, 0.033 *M* potassium succinate and ferricytochrome c (about 0.26×10^{-4} *M*). After the addition of a suitably diluted rat liver homogenate (0.1 ml), the increase in optical density at 550 *mμ*

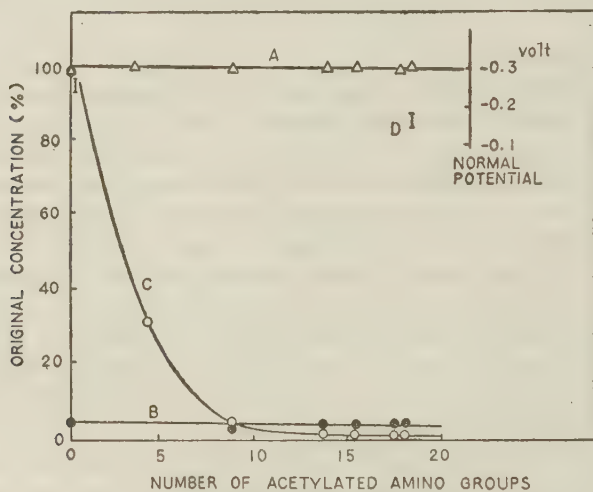


FIG. 2. Acetylation of cytochrome c.

A: the intensity of the absorption at 550 *mμ* in the reduced state, B: the affinity towards carbon monoxide in the reduced state, C: the electron transferring ability in succinic oxidase system, D: the oxidation-reduction potential.

was observed either aerobically in spectrophotometer cuvette (reaction mixture 3.0 ml) or anaerobically in Thunberg tube by using a hand-spectroscope. When a hand-spectroscope was used, the absorption at 550 *mμ* was compared with standards of known concentrations of completely reduced cytochrome c.

Enzymatic oxidizability was estimated spectrophotometrically by the disappearance of the absorption at 550 *mμ* after the addition of liver homogenate to the solution of reduced cytochrome c. When the autoxidation of the derivative was pronounced, the same solution without addition of the homogenate was followed as control.

In acetylated cytochrome c was observed the decrease of both oxidizability and reducibility in enzymatic system. As shown in Fig. 3 and Table I, the enzymatic reduction became sluggish as the acetylation progressed.

However, the extent of the enzymatic reduction in anaerobic state was observed to be complete and the fully reduced spectrum appeared after several hours of the incubation. The speed of the oxidation of acetylated derivative by cytochrome oxidase became slow and the speed of the autooxidation became faster in parallel with this.

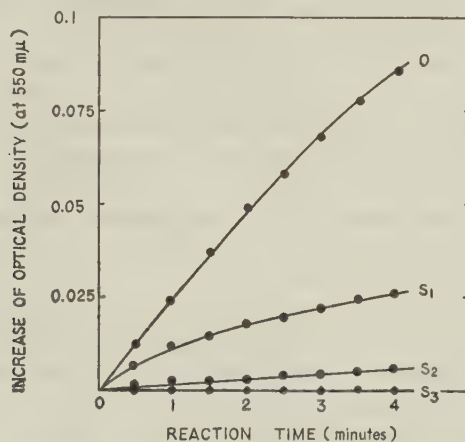


FIG. 3. Enzymatic reduction of acetylated cytochrome c in aerobic condition. Percentage of acetylated amino groups: O (control); S₁, 78.5; S₂, 85.6; S₃, 87.6.

TABLE I

Time Necessary for Enzymatic Reduction of Acetylated Cytochrome c in Anaerobic Condition

Sample No.	1	2	2	4	5
Percent of acetylated amino groups	0	15	28	78	85
Time necessary for 90 per cent reduction	15 min.	1 hr.	2.5 hrs.	10 hrs.	15 hrs.

Ascorbic Acid Oxidase Activity—This activity was estimated manometrically at 37° in 0.065 *M* phosphate buffer pH 7.4, with 0.025 *M* sodium ascorbate.

The significant increase of ascorbic acid oxidase activity was not observed in acetylated derivative.

Hydroxylamine Reductase Activity—This activity was estimated by measuring the time necessary for the decolorization of reduced methyl viologen using Thunberg tubes at 30°. Reaction mixture; 0.023 *M* phosphate buffer pH 7.0, 1×10^{-3} *M* reduced methyl viologen, 0.03 *M* hydroxylamine hydrochloride, and 3×10^{-7} to 10^{-9} *M* cytochrome c.

The significant increase of hydroxylamine reductase activity was not observed in acetylated derivative, as well as well as ascorbic acid oxidase activity.

Oxidation-reduction Potential—For the measurement of oxidation-reduction potential, ferro-ferri cyanide oxidation-reduction buffers (10) and oxidation-reduction dyes (11) were used.

Slight fall of normal potential of the derivative was observed as the extent of the acetylation proceeded. The reduced spectra of the completely acetylated derivative appeared on titrating with sodium dithionite solution, just before the decolorization of 2,6-dichlorophenol indophenol and the potential was estimated to be about +0.15 volt. This fall was insignificant when compared with that of protease digestion (−0.3 volt) (11).

Solubility and Other Properties—Acetylated cytochrome c is soluble in water. However, the solubility in salt solution is diminished and the precipitation is caused in half saturated sodium acetate solution when the acetylation proceeds to a certain degree. The precipitation was also observed when acidified, whereas native cytochrome c did not precipitate even in 1 *N* hydrochloric acid.

Another remarkable feature in acetylated cytochrome c was that the pigment was not absorbed on a dialysis membrane even when dialysis was done against distilled water. The absorption of the native pigment on cellophane is so remarkable, that Theorell used the absorption on cellophane at one of the purification stage of cytochrome c (12).

These observations will be explained by the loss of positive electronic charges due to the blocking of amino groups.

DISCUSSION

The above findings show the importance of amino groups for the function of cytochrome c. Acetylated cytochrome c did not accelerate oxygen uptake in succinic oxidase system. This does not mean, however, that amino groups are responsible directly for the electron transfer. As shown from the experiment of enzymatic reduced and oxidized completely only after longer interval but appeared to be inactive in succinic oxidase system if measured manometrically. The necessary conditions for a electron transferring mediator, the maintenance of oxidation-reduction potential in a certain range and reactivities with reductase and oxidase, must have been fulfilled. Thus, acetylated cytochrome c has the ability to mediate electron transfer in biological system, but is less efficient as the native one. It may be possible to say, that acetylated derivative of cytochrome c is analogous to certain oxidation-reduction dyes in respect to the ability of electron transfer. The efficiency of oxidation-reduction is necessary for the actual participation in biological electron transfer.

These findings remind the authors the supposition stated by Keilin (13), that cytochrome c as it exists in cell is hundred times more efficient than the extracted and purified one. Some sorts of modifications other than the acetylation will cause a decrease of the efficiency in electron transfer, even if there modified ones can be used as an electron transferring mediator.

By treating cytochrome c with *N* potassium hydroxide, Keilin and

Hartree (14) obtained an autoxidizable cytochrome c which was catalytically inactive in biological systems. Thereafter, Tsou (8) also obtained an autoxidizable fragment by proteolytic digestion. He observed that the fragment combined with carbon monoxide in the reduced state, and recommended a spectrophotometric estimation of an autoxidizable and biologically inactive cytochrome c by the carbon monoxide method.

However, the fact that the autoxidizability is not always accompanied by the carbon monoxide binding affinity was shown in the acetylation of cytochrome c. That is, a biologically inactive cytochrome c obtained by the acetylation was autoxidizable, but did not combine with carbon monoxide at all. From this finding, it is indicated that the estimation of an autoxidizable and biologically inactive cytochrome c by Tsou's carbon monoxide method is not applicable in certain cases.

The characteristic features of acetylated cytochrome c differing from the protease-digested one are that the both ascorbic acid oxidase and hydroxylamine reductase activities were negligible compared to the vigorous rise of both activities seen in the case of protease digestion.

The main feature of the acetylation is that the positive electric charges of amino groups are lost. This may cause some changes in the stereochemical configuration of protein and may be serious in the case of cytochrome c in which lysine content amounts to as much as 20 per cent of amino acid residues. A somewhat specific stereochemical configuration seems to be necessary for the efficiency of oxidation-reduction and non-autoxidizability of the pigment. The guanidination of cytochrome c was also undertaken in the authors' laboratory (15). Nearly complete guanidination of amino groups, that is only replacement of amino groups with similarly basic guanidino groups, did not cause any loss of activity in succinic oxidase system. This observation confirms the above supposition.

SUMMARY

1. Cytochrome c was acetylated with acetic anhydride and electrophoretically homogeneous derivatives acetylated to several degrees were obtained.

2. Acetylated cytochrome c had several physicochemical properties different from the native one due to the loss of positive electric charges: electrophoretic mobility, non-absorption on resin IRC-50 or cellophane dialysis membrane and the diminution of solubility in acidic medium.

3. The absorption spectra were not modified by the acetylation. Acetylated cytochrome c was readily autoxidizable in air but did not combine with carbon monoxide.

4. Acetylated cytochrome c did not accelerate oxygen uptake in succinic oxidase system if measured manometrically. However, it could be oxidized and reduced enzymatically, though the efficiency was reduced by the treatment.

5. Ascorbic acid oxidase and hydroxylamine reductase activities were

negligible in the acetylated derivative.

The authors wish to express their thanks to Prof. S. Akabori and Prof. H. Yoshikawa for constant guidances in the course of the work. Thanks are also due to Prof. Y. Yoneyama and Dr. M. Ishimoto for their valuable discussions.

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TURBIDOMETRIC TITRATION OF SERUM-PROTEIN

II. SPECIES SPECIFICITY OF SERUM ALBUMIN* MASARU SOGAMI

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(Received for publication, August 14, 1957)

Recently, methods detecting very subtle differences between similar proteins have been developed. Therefore, numerous investigations have been carried out during past decades dealing with species specificity of serum albumin. Methods in general use were as follows; (A) immunological reaction (1, 2), (B) analysis of amino acid composition (3-6), and (C) electrophoretic method (7-9). And also, these methods described above have been used for the investigation of microheterogeneity of protein, since these were very sensitive (6).

On the other hand, the author has investigated the salting out of protein and has devised turbidometric titration of protein solution (10). This method was consistent with the turbidometric titration of synthetic high polymer solution which has been used for determination of molecular weight distribution function of polymer (12-15). Then, the author discovered that turbidometric diagram of serum albumin had many peaks (10) and that the author's method was capable of detecting specificity of serum albumin.

In the present paper, the author showed the species difference of serum albumin under three headings: (I) species difference in electrophoretic patterns of serum protein (II) reproducibility of turbidometric diagram of serum albumin and (III) species difference in turbidometric diagram of serum albumin.

METHOD AND MATERIALS

Following the previous reports (10) turbidometric titration was carried out by using ammonium sulfate solution as a precipitant and K-phosphate buffer (100 ml. of 0.01 M K-phosphate buffer, pH 7.35, +900 mg. of NaCl) as a solvent (16). While in the previous reports (10) protein concentration was about 0.3 per cent, in this report it was about 0.03 per cent. However, both results were the same except for some region which was highly saturated with precipitant. And then, a previously reported disturbance of turbidometric titration of protein solution (10) was eliminated by lowering the protein

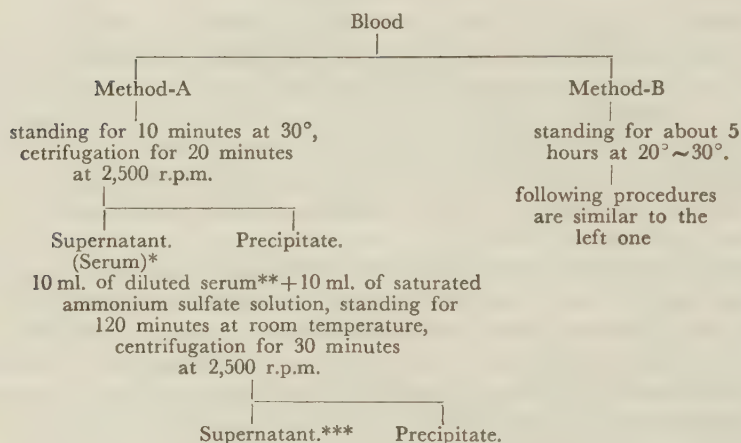
* These data were represented at Kinki Branch Meeting of physiological Society of Japan at Osaka Dental College in February, 1957.

concentration.

Paper electrophoretic analysis was made on 0.02~0.025 ml. of serum in 0.045 μ veronal buffer at pH 8.5 (17) according to Grassmann's method (12, 18, 19). Five 30 $\times$ 365 mm. strips a (Toyo filter paper No. 131) were ran simultaneously in one cell for 10 hours at 6~10° under constant current condition of 0.54 mA per 1 cm. Constant current condition* of 0.54 mA per 1 cm² was used because of low temperature of the room. The strips were stained by using brom phenol blue (20), and electrophoretic patterns were recorded by transmission densitometry. And also, serum used in electrophoretic analysis was prepared by using method A illustrated in Scheme 1.

SCHEME 1.

Schematic Interpretation of Sample Preparation Used in Turbidimetric Analysis of Serum Protein



* Serum was used for the paper electrophoretic analysis.

** For turbidimetric analysis, serum was diluted approximately from 200 to 300 folds with K-phosphate buffer (100 ml. of 0.01 M K-phosphate buffer pH 7.35, +900 mg. of NaCl).

*** Supernatant liquid was titrated with saturated ammonium sulfate solution.

EXPERIMENTAL RESULTS

(I). *Paper Electrophoresis of Serum Protein*—Results on on paper electrophoretic patterns of human, rabbit and goat serum protein were shown in Fig. 1. And also, results on human plasma protein, serum albumin**, serum γ -globulin** and bovine plasma albumin were shown in Fig. 2.

* The apparatus for constant current was designed by Dr. H. K i h o, Dr. K. K a w a i (Department of Physics Faculty of Science, Kyoto University) and the author, and was made by the author, Dr. K. T o r i z u k a and Assist. Prof. G. W a k i z a k a (Department of Internal Medicine, Faculty of Medicine, Kyoto University).

** These samples were kindly given by Dr. O. N a k a g a w a (Nippon Med, Co., Tokyo, Japan).

As shown in Figs. 1 and 2, serum protein from different species was fractionated into the following many components; human serum protein into albumin, α_1 , α_2 , β , γ -globulin, rabbit serum into albumin, α_1 , α_2 , β_1 , β_2 ,

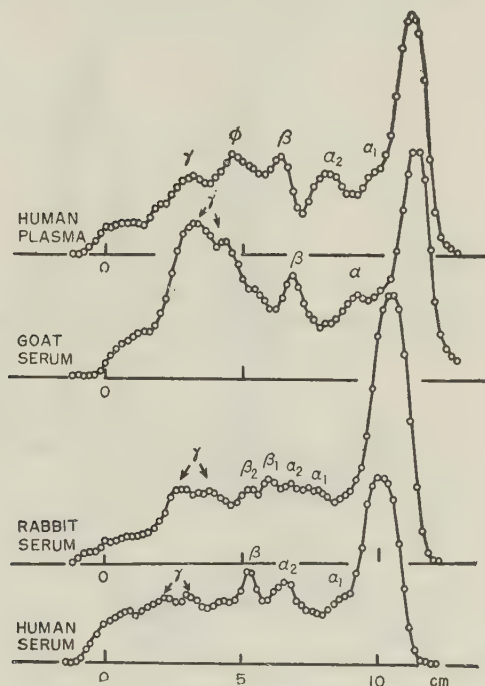


FIG. 1. Paperelectrophoretic patterns of human plasma, goat serum, rabbit serum and human serum in veronal buffer (pH 8.50, ionic strength 0.045). See text for other electrophoretic condition.

γ -globulin and goat serum into albumin, α , β , γ -globulin. Then, γ -globulin was fractionated into 2 components under the author's experimental conditions, namely in veronal buffer (pH 8.5, $\mu=0.045$), 0.54 mA/cm. However, using the same buffer, zone electrophoretic experiment with potato starch column by the method of Kunkel (21) showed that human and rabbit γ -globulins were not fractionated into 2 components under constant current conditions of 2.0 mA/cm² or 3.2 mA/cm².

As described above, the result on serum protein from different animals indicated the significant difference in serum protein composition. These findings presented here were not inconsistent with other reports (22). However, paper electrophoretic patterns of human serum albumin and bovine plasma albumin did not indicate any significant difference as shown in Fig. 2.

(II). *Reproducibility of Turbidometric Diagram*—The author investigated the reproducibility using the same goat serum albumin and showed one example in Fig. 3. In order to obtain reproducible results, "the effect of temperature on serum separation" (See next section) should be considered.

(III). *Species Specificity of Turbidometric Diagram of Serum Albumin*—Serum

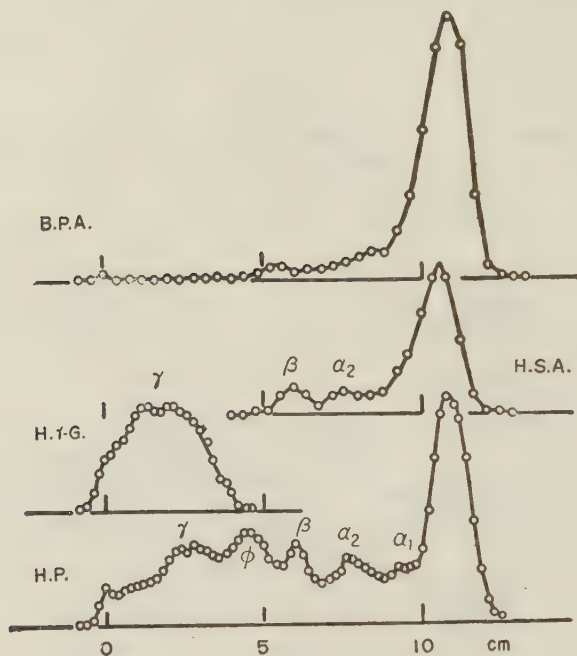


FIG. 2. Paper electrophoretic patterns of human plasma, human serum albumin, human γ -globulin and bovine plasma albumin in veronal buffer (pH 8.50, ionic strength 0.045). See text for other electrophoretic conditions.

albumin of man, goat and rabbit, and bovine plasma albumin were used in this experiment. Serum used in this experiment was prepared by using method A illustrated in Scheme 1.

Then, turbidometric diagram of albumin had many peaks as shown in Figs. 4 and 5. For instance, bovine plasma albumin had seven peaks. (paper electrophoretic diagram of bovine plasma albumin was shown in Fig. 2). Results on man, rabbit, goat and beef indicated satisfactory significant differences in serum albumin composition. Namely, human, rabbit and goat serum albumin, and bovine plasma albumin had their highest peaks at j_2 , k_2 , l m, and n_2 , respectively.

"The effect of temperature on serum separation" should be taken into account in order to obtain accurate result. For instance, using method A or B, turbidometric diagrams of serum albumin from different species had

their highest peaks at the following positions: human serum albumin* at j_2 or k_1 (11), rabbit serum albumin at k_2 or k_1 as shown in Fig. 5. However, turbidometric diagram of goat serum albumin always had highest peaks at

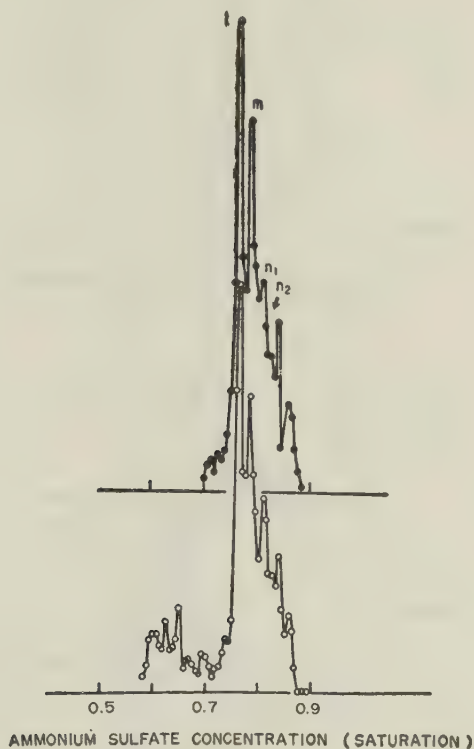


FIG. 3. Turbidometric diagram of goat serum albumin. The abscissa and the ordinate show volume fraction, C , of saturated ammonium sulfate solution and $\Delta T / \Delta C$, respectively, where T shows relative amount of salted-out protein. Experimental conditions were as follows; solvent K-phosphate buffer (100 ml. of 0.01 M K-phosphate buffer, pH 7.35, +900 mg. of NaCl), pH 6.2 at 40 per cent saturation and pH 5.95 at 70 per cent saturation, protein concentration about 0.03 per cent.

1 and m as shown in Fig. 5.

* Solubility curve of human serum albumin was further modified by passage through Dowex-2 anion exchanger column. This may be due to the removal of small molecule from serum albumin (29).

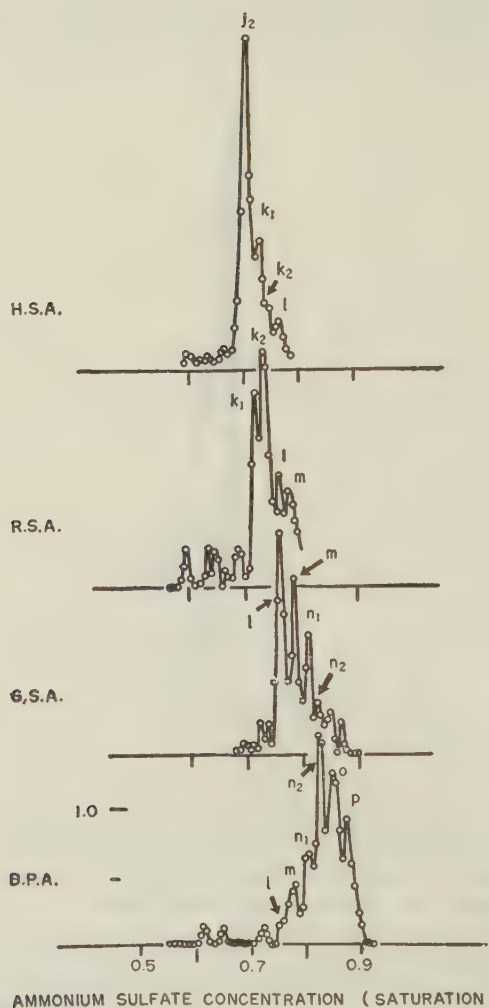


FIG. 4. Turbidometric diagram of human, rabbit, goat serum albumin and bovine plasma albumin. The abscissa and the ordinate show volume fraction, C , of saturated ammonium sulfate solution and $\Delta T/\Delta C$, respectively, where T shows relative amount of salted-out protein. Experimental conditions were as follows; solvent K-phosphate buffer (100 ml. of 0.01 M K-phosphate buffer, pH 7.35 + 900 mg. of NaCl), pH 6.1 at 40 per cent saturation and 5.95 at 70 per cent saturation, protein concentration about 0.03 per cent.

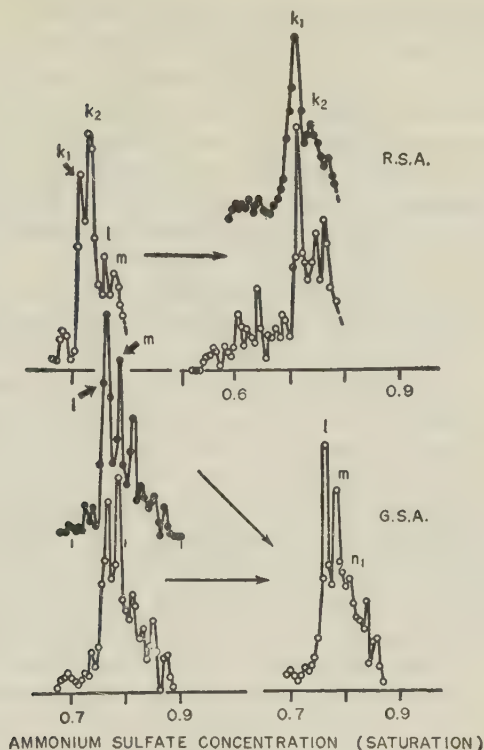


FIG. 5. Turbidometric diagram of rabbit and goat serum albumin fraction. The abscissa and the ordinate show volume fraction, C , of saturated ammonium sulfate solution and $\Delta T/\Delta C$, respectively, where T shows relative amount of salted-out protein. Material of left and right figures were prepared by the use of method A and B (See Scheme 2), respectively. Other experimental conditions were as follows; solvent K-phosphate buffer (100 ml. of 0.01 M K-phosphate buffer, pH 7.35, +900 mg. NaCl), pH 6.2 at 40 per cent saturation and 5.95 at 70 per cent saturation, protein concentration about 0.03 per cent.

DISCUSSION

In the last section, the author described the species specificity of albumin and whole serum protein using turbidometric analysis and paper electrophoretic analysis. In this section, the author made a discussion comparing the author's results with those of other investigators.

(A) Melcher *et al.* (1) investigated the species specificity using immunological method, and discovered that cross reaction of bovine serum albumin with anti-human serum albumin was about 15 per cent. And also, Mauea *et al.* (2) investigated the species specificity of serum albumin using

the same method, and discovered that cross reaction of serum albumin were as follows: bovine serum albumin with anti-human serum albumin 3 per cent, human serum albumin with anti-bovine serum albumin 6 per cent, bovine and human serum albumin with anti-rabbit serum albumin 0 per cent. And also, he discovered that there was greater similarity in antigenic groupings among γ -globulin than among albumin. In contrast to these results, in turbidometric diagram, there was greater difference between human and bovine serum albumin than between human and rabbit serum albumin. And also, in turbidometric diagram, there was little difference between human and rabbit γ -globulin (23). However, in turbidometric diagram of γ -pseudoglobulin, there was significant difference between human and rabbit (23).

Difference in amino acid composition of serum albumin from different species have been reported by Kusama (3), Hughes (4), Tristram (5). Kusama investigated C-terminal amino acid residue and discovered the following interesting results on serum albumin: leucine (man, rabbit) and alanine (goat, sheep and beef). In turbidometric diagram, there was greater difference among human, goat serum albumin and bovine plasma albumin than between human and rabbit serum albumin.

The electrophoretic difference of serum albumin from different species have been carried out by Landsteiner (7), Alberty (8) and Saifer (9). At lower pH regions than isoelectric point of serum albumin, electrophoretic patterns of serum albumin showed the species specificity. Namely, Alberty (8) reported that bovine albumin was resolved into three components at pH 4.15 but human albumin into only two components. Saifer *et al.* (9) reported that human serum albumin was resolved into four components in acetate-NaCl buffer at pH 4.0, ionic strength 0.05 and that there was significant difference among bovine, human and porcine serum albumin. While these electrophoretic analysis was carried out in acidic solution, turbidometric titration was carried out at higher pH than isoelectric point of serum albumin. Therefore, the author investigated pH dependence of turbidometric diagram of human serum albumin (11) and discovered that there were more peaks at pH 3.9 than at pH 6.0. Therefore, turbidometric titration at lower pH region may be capable of detecting more subtle difference between similar proteins, but may be cumbersome because of necessity of purification of serum albumin. As described above, turbidometric diagram of serum albumin from different species indicated significant difference. However, the mechanism by which species specificity appears in turbidometric diagram will not be clear until more information is available on the turbidometric diagram (29).

Chromatographic analysis (24-26), and Derrien's method (27) may be very effective to investigate the species specificity of serum albumin. On the other hand, Momotani has investigated turbidometric titration of seed proteins and has discovered the species specificity (28).

(B) Paper electrophoretic results on serum from different species also

indicated some differences, as reported by many investigators (22).

SUMMARY

The author investigated the species specificity of serum albumin and serum of man, rabbit goat and beef, using turbidometric titration and paper electrophoresis. The results were as follows.

1. The turbidometric titration of serum albumin had many peaks and allow to detect very subtle differences (as species specific) among different species (Fig. 4).

2. In order to obtain accurate turbidometric diagram, special care must be payed for the experimental conditions of serum separation, especially on the temperature influence upon the turbidometric diagram of serum albumin as illustrated in Scheme 1 and Fig. 5.

3. Paper electrophoretic patterns of serum showed differences among man, rabbit and goat.

The author wishes to thank Prof. K. Sasagawa and Assist. Prof. Y. Tamura (Department of Physiology, Faculty of Medicine, Kyoto University) for their advice to my investigation. And also, the author wishes to thank Prof. T. Yamamoto Dr. M. Yamamoto (Department of Quantum Chemistry, Faculty of Science, Kyoto University), Prof. G. Wakizaka, Dr. K. Torizuka (Department of Internal Medicine, Faculty of Medicine, Kyoto University), Dr. H. Kiho, Dr. K. Kawai (Department of Physics, Faculty of Science, Kyoto University) and Dr. Y. Momotani (Department of Botany, Faculty of Science, Kyoto University) for their excellent discussion.

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ACRIDINE SALT OF FLAVINMONONUCLEOTIDE

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(Received for publication, December 3, 1957)

In 1936 Wagner-Jauregg (1) prepared the characteristically crystallized acridine salts of AMP*, ADP, and ATP by means of either adding an aqueous solution of these free nucleotides to an alcohol solution of acridine, or mixing a weak acidic solution of barium salts of these nucleotides with acridine hydrochloride. In 1949 Todd *et al.* (2) applied such an acridine derivative for the purification and identification of the synthesized ATP, and found that the melting point of triacridinium adenosine-5'-triphosphate prepared from its crude preparation well agreed with that of the pure ATP from the natural source. And in 1952 Karrer *et al.* (3) also prepared the acridine salt of pridoxal phosphate from its crude preparation.

The crystallized acridine salts of the flavin nucleotides such as FMN and FAD, which possess definite melting points, will be also expected to prepare and to serve for their purifications and identifications.

It is the purpose of this paper to present the preparation of acridine salt of FMN.

EXPERIMENTAL

Preparation of Free FMN—To the sodium salt of FMN (Hoffman-La Roche Co.) added the calculated volume of *N* HCl followed by the addition of 5 volumes of absolute alcohol. The resulting precipitate was collected by the centrifugation and dried *in vacuo*. Free FMN was thus obtained.

Purification of Acridine—Acridine (Tokyo-kasei Co.) was recrystallized thrice from alcohol-water and then dissolved in benzene. This benzene solution was passed through an alumina column and then benzene was evaporated off. The residue was recrystallized from alcohol-water. White crystals (orthorhombic plates) were obtained. m.p. 113°.

Preparation of Acridine Salt of FMN—To a solution of 106 mg. of FMN

* The following abbreviations are used: adenoside monophosphate, AMP; adenosine diphosphate, ADP; adenosine triphosphate, ATP; flavinmononucleotide, FMN; flavin-adeninedinucleotide, FAD.

in 20 ml. of hot distilled water, a solution of 39.2 mg. of acridine in 4 ml. of hot absolute alcohol was added dropwise. Cooling gradually to the room temperature, the mixture gave a deep yellow solution but deposited no crystals. After preserving this solution in the dark for 10 days, a mass of

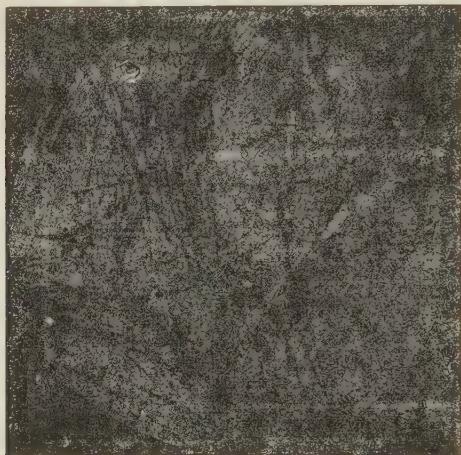


FIG. 1. Acridine salt of FMN.

orange yellow crystals appeared leaving the decoloured mother liquid (*cf.* Fig. 1). After 4 days these crystals were collected by the filtration, washed with a small volume of absolute alcohol, and recrystallized from hot distilled water. 47.5 mg. of brownish yellow needles were obtained. The dried substance had a melting point of 217° with previous darkening and showed following analysis.



Calcd.: C, 55.89; H, 4.85; N, 10.87 per cent

Found.: C, 56.14; H, 4.93; N, 10.71 per cent

This substance was soluble in boiling water but insoluble in cold water, alcohol, and ether.

Separation of FMN from This Acridine Salt—About 3 mg. of FMN-acridine suspended in 2 ml. of water and pH was adjusted to 8.5 with *N*/50 NaOH. Free acridine separated flocculently from the yellow solution. When this turbid yellow solution was shaken with ether, acridine was transferred into the ether layer. After washing several times with ether the water layer was diluted with alcohol giving a yellow precipitate of free FMN, which was chromatographically pure.

The author wishes to express his sincere appreciation to Prof. K. Makino and Instructor K. Satoh for their valuable guidance and kind encouragement. FMN was kindly supplied by Wakamoto Pharmaceutical Co. Ltd., and the analytical data in this paper due to the kindness of analytical laboratory, Sankyo Pharmaceutical Co. Ltd. This work was supported by a grant from the Vitamin B Research Committee

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MALONATE METABOLISM IN HUMAN PLACENTA

Using human chorionic slice (9-12 weeks of placenta: dry weight *ca.* 30 mg.), authors discovered that malonate (final concentration $10^{-2} M$) was not an inhibitor but increased oxygen consumption for endogeneous respiration in Warburg's flask (total volume 3.0 ml.). On adding malonate to succinate, however, the level of the oxygen consumption increased by succinate fell down to the level of malonate alone.

Using human chorionic homogenate, it was also found that malonate ($100 \mu M$) increased oxygen consumption (Table I). When malonate was

TABLE I
*Oxygen Consumption in Chorionic Homogenate with Addition of
Succinate, Malonate and Fumarate*

	Succinate	Succinate Fumarate	Succinate Malonate	Malonate	Fumarate	No addition
$O_2 \mu l.$ /mg. N/hr.	21	17	16	15	14	11

Incubation mixture (3.0 ml.) contained 1.0 ml. of 20 per cent chorionic homogenate in 0.25 *M* sucrose (N content about 3.0 mg.), 2.5 μM of ATP, 0.75 μM of DPN, 0.01 μM of cytochrom c, 30 μM of K^+ , 10 μM of Mg^{++} , 20 μM of Cl^- , and 20 μM of phosphate in main flask, 50 μM of each substrate in side arm, and 0.2 ml. of 10 *N* KOH in center well. pH 6.9, 37°. Gas phase air.

added to succinate, malonate caused the decrease of oxygen up-take to the level of malonate alone. In Thunberg's tube it was observed that succinic dehydrogenase activity in chorionic homogenate was inhibited competitively with malonate.

Fumarate increased oxygen up-take, but, in the presence of succinate, the addition of fumarate caused the decrease of the oxygen up-take to the half level between succinate and fumarate. Fumarate has been assumed to be an inhibitor of succinic dehydrogenase and is metabolized easily as a member of TCA-cycle. Thus, malonate shows the similar behaviour to fumarate in manometric study. Though placenta contains much blood, the increase of oxygen up-take with malonate was not observed in the case of erythrocytes or hemolyzed blood.

The oxygen consumption of chorionic homogenate increased parallel to the increase of the concentration of malonate, and the linear relationship was observed in the range of 0-100 $\mu g.$ of malonate concentration. When malonate was added after the previous incubation of the homogenate for 30

minutes, the increasing curve of oxygen up-take followed the curve obtained by addition of malonate from the beginning.

Anaerobic study was undertaken to investigate the metabolism of malonate by human chorionic homogenate and to test the possible occurrence of the mechanism observed in microbes (1, 2) also in the degradation by mammalian tissues. For the decarboxylation of malonate, ATP, Mg^{++} and CoA were needed (Table II), and the linear relationship was observed

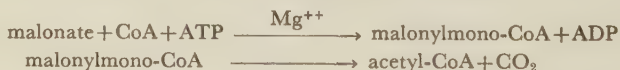
TABLE II
Decarboxylation of Malonate in Human Chorionic Homogenate

	Complete System	Except ATP	Except CoA	Except $MgCl_2$	Except ATP CoA $MgCl_2$
CO_2 $\mu l./30$ mins.	80	32	64	55	13

Incubation mixture (3.0 ml.) contained 1.0 ml. of 20 per cent human chorionic homogenate (aged for 4 hours in refrigerator), 50 μM of Tris buffer (pH 7.0), 100 μM of malonate, 5 μM of $MgCl_2$, 0.3 μM of CoA and 5 μM of ATP. Incubation was carried out for 30 minutes at 37° in 95 per cent N_2 +5 per cent CO_2 , after which 100 μM of H_2SO_4 was tipped in.

between time interval and carbon dioxide production. The involvement of ATP, CoA and Mg^{++} in the decarboxylation of malonate suggests that a CoA-activated malonate is formed during the course of the reaction. The identification of malonyl-CoA was carried out by preparing malonylhydroxamate ester from it, which was checked by paper chromatography (3). Two hydroxamate spots were obtained from the extract of the incubation mixture and their R_f values were 0.55 and 0.33. These R_f values correspond to the R_f values for malonylmonohydroxamate and acetylhydroxamate.

The mechanism for malonate degradation appears to be similar to that found in bacteria and could be formulated as follows:



During the course of this study, a paper on the extent and pathway for malonate degradation in mammalian system appeared, studied independently by Nakada, Wolfe and Wick (4) in rat tissues.

This report was presented at the 29th and 30th meetings the Japanese Biochemical Society on Nov. 1, 1956 and July 16, 1957.

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